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(54) Title: METHODS AND COMPOSITIONS FOR IMPROVING THE SUCCESS OF CELL TRANSPLANTATION IN A HOST

(57) Abstract

The present invention covers significant improvements for each event involved in the transplantation success or graft survival. These improvements, separately or combined with each other, greatly ameliorate the recovery of a tissue towards a normal function. They comprise: a) the reduction of early death of transplanted cells by anti-inflammatory agents such as TGFbeta1, an inhibitor of oligosaccharide synthesis, a glucosidase, IL-10, vIL-10, IL-4, INFgamma, IL-2R, IL-1Ra, Fas-L, sCR1, a super oxide dismutase, a neutrophil inhibitory factor (NIF), a ligand binding in an antagonist fashion to LFA-1, MAC-1, ICAM-1, CD-18, CD-31, CD-50, E-selectin, P-selectin, TNFalpha, IL-1 and IL-8. The anti-inflammatory agents may comprise an anti-LFA-1 or -ICAM-1; b) the improvement of the diffusion and of the fusion of transplanted cells with the host tissue by metalloproteases; c) the *ex vivo* proliferation of the transplanted cells with growth factors or oncogenes; d) the use of fibroblasts or stem cells in lieu of myoblasts, by transforming the formers into the latter with myogenic genes; e) expressing utrophin in lieu of dystrophin in cases of muscular dystrophy; and e) immunosuppressing the host for long-term graft survival.

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TITLE OF THE INVENTION

Methods and compositions for improving the success of cell transplantation in a host.

FIELD OF THE INVENTION

This invention relates to a method of restoring a functional protein in a tissue by transplanting donor cells or genetically modified donor or host cells, the method comprising one or more of the following the steps : a) reducing the early mortality by way of an anti-inflammatory therapy, b) increasing the diffusion of surviving donor cells into the host tissue, c) injecting a greater amount of donor cells already grown and multiplied *in vitro* and injecting cells which have been stimulated to proliferate or preconditioned for survival, d) increasing the fusion between the donor and host cells, and e) undertaking a suitable immuno-suppressive therapy.

BACKGROUND OF THE INVENTION

Cell transplantation is a potential treatment for several diseases. Myoblast transplantation may indeed be used to treat Duchenne Muscular Dystrophy (Partridge 1991), heart insufficiency, nanism (Dhawan et al. 1991), hemophilia (Dai et al. 1992) and Parkinson (Jiao et al. 1993); neuron transplantation may be used to treat Huntington disease (Emerich, 1995) and Parkinson disease (Borlongan and Sandberg, 1995); islet may be used for diabetic (Hering et al. 1993), hepatocytes for liver diseases (Raper and Wilson, 1993). The success of these cell transplantations has been so far rather limited. This limited success has been attributed in part to the specific immune responses (Huard, 1992, Tremblay, 1993). However, some research groups have also reported high levels of cellular death during the first three days following cellular transplantations (Beauchamps et al. 1994; Huard et al. 1994). This time course is too rapid to be attributed to a specific immune response in naive animals.

The principal problems to overcome during cell transplantation are the following:

a) a host specific immune reaction towards donor cells;
b) the number of cells expressing the non-defective protein;
c) the diffusion of donor cells in the recipient tissue;
d) the amount of non-defective protein expressed in the cells participating to tissue function recovery; and
e) the high donor cell death rate.

Although solutions to some of these problems have been proposed in the prior art, the perfect methods and compositions are still to come.

a) <u>A host immune response towards donor cells:</u>

It is now known that an adequate immunosuppressive therapy should be undertaken in the case wherein host and donor are histoincompatible, to avoid

rejection of the transplanted cells. Shortly after transplantation, infiltrating cells may be observed at the site of transplantation. Even in the case wherein histocompatible host and donor are selected (matched for MHC class I and class II antigens), other non-MHC antigens might be responsible for an observed immune response. Although the non-specific inflammatory response decreases with time, the specific cellular and humoral response starts building after one week and may be largely responsible for the lack of long-term success of transplantation, especially in immunohistoincompatible subjects.

Nonspecific immunosuppression has been found successful for improving and maintaining the success of transplantation. Immunosuppressors like FK506, rapamycin, cyclosporine A and cyclophosphamide did improve the survival time of the transplant. Among these, cyclosporine A, FK506 and rapamycin have been found the most potent. Since immunosuppressors provoke undesirable side effects, it is highly desirable to match histocompatible subjects and to use a more specific immunotherapy, when needed or to genetically modify the host's own cells. With adequate immunosuppression, it may be possible to transplant cells originating from a different species, for example normal or genetically modified pig myoblasts could be transplanted into human muscles to restore a defective protein.

Histocompatibility is not the only factor to consider for increasing the success of transplantation. When myoblasts from identical twins are transplanted, there is still a poor regeneration of muscle function. It appears to be due to the low spontaneous muscle regeneration or to inflammation.

b) <u>The number of cells expressing the non defective protein:</u>

The number of cells expressing a non-defective protein, e.g., fused donor cells or hybrid donor-recipient muscle fibers, are usually quite low in non-immunosuppressed individuals. Beside the beneficial effect of an adequate immunosuppressive therapy or of an adequate match of donors and recipients on the number of functional cells, other strategies have been used to increase the production of non-defective proteins. It has been found that pre-treating recipient *mdx* mice (an animal model for Duchenne muscular dystrophy) with notexin and radiation, which has for effect to destroy the capability of the recipient muscle cells to proliferate, gives a much better chance to the donor cells to proliferate and to reconstruct a functional tissue. Up to recently, the best results have been obtained by combining immunosuppressive and tissue treatment with notexin and irradiation. However, notexin and irradiation remain unacceptable from a clinical point of view because they have severe undesirable side effects. In the international patent publication WO 96/28541, a pre-treatment step consisting of culturing myoblasts in the presence of a trophic factor is described. Particularly, the obtention of primary cultured myoblasts in the presence of bFGF (basic fibroblast growth factor) is shown to increase by four-fold

the number of dystrophin-positive cells. This increase is almost equivalent to the one resulting from a notexin treatment. This increased success has been attributed to a stimulation of the myoblasts by bFGF which increases either the surviving capacity *in vivo* or their proliferative capacity *in vivo* during the first few days following transplantation. This represents a clear progress to enhance the success of transplantation in absence of notexin and irradiation pre-conditioning step in dystrophic individuals. Even with this progress, the level of cell mortality still remains very high (75 to 99%) unless an anti-inflammatory therapy is undertaken from the very first day of transplantation. It is therefore important to transplant donor cells which have the best survival and proliferative potential.

c) <u>The diffusion of donor cells in the recipient tissue:</u>

The diffusion of transplanted cells into the recipient tissue is another important problem. In the international patent publication WO 98/17784, I described the use of metalloproteases, namely matrilysin and gelatinase A, and of agents capable of inducing the formation and secretion of metalloproteases, such a concavanalin A and phorbol esters, for increasing the number of fused donor/host myoblasts and for increasing the surface of the muscle capable of expressing a marker protein. It is worthwhile recalling that in patients suffering of muscular dystrophy, the extra-cellular matrix is more developed than normally. The extra-cellular matrix represents a barrier to the diffusion of transplanted cells, and metalloproteases have been proved to be tool to moderately favor the diffusion of donor cells into the recipient tissue. The most spectacular results from metalloproteases are found in the increase of the rate of fusion between donor and host cells which participate in the recovery of a normal muscular function. An alternative way to resolve the limited diffusion of myoblasts (instead of genetically introducing metalloproteinase genes) is to do very closely spaced myoblast injections (1 to 1.5 mm apart). Such close injection trajectories may be done by using a robotic device controlled by a computer receiving information from an imaging system such as NMR (Nuclear Magnetic Resonance), echography system or low level X ray system such as those used for catheterism. To overcome the diffusion problem, it may also be possible to transplant the cells by arterial or venous injections. The cells would have to be genetically modified or stimulated with cytokines to attach to the endothelial cells of the blood vessels. The secretion of metalloproteinases by the genetically modified cells may permit them to cross the blood vessels and invade the tissue.

d) <u>The amount of non-defective protein expression in the cells participating to tissue function recovery:</u>

Although a high percentage of cells which survive after one week of transplantation expresses a non-defective protein (about 90% expressing dystrophin in an *mdx* mouse), the level of expression of a functional protein (dystrophin) per cell

remains quite variable. There is therefore room for improving the level of expression of a non-defective protein in transplanted or fused cells specially when the transplanted cells are the host own cells genetically modified to express a protein lacking or defective in this patient.

e) <u>The high death of transplanted cells:</u>

This problem was one of the most serious to overcome until recently. Immunosuppressive treatment has been successful in inhibiting the specific cellular and humoral immune response which appears later than the non-specific inflammatory response. However, there was still a very high early mortality of the transplanted cells (75 to 99%). Inflammation may be one of the major factors involved in the important early mortality, e.g., before the building of a cellular or humoral response. Inflammation may be also partly responsible for the poor success of muscle regeneration observed after the transplantation of myoblasts between identical twins.

Despite all the attempts made to improve the success of transplantation, there was still a need for increasing the number of cells which survive after the first three to seven days of transplantation, beyond which immunosuppressive treatments as well as an enhanced proliferation of the cells which have survived may exert their beneficial effects to improve long-term recovery of tissue function.

In the international patent publication WO 97/36602, I described the use of an antibody directed against either LFA-1 blood cell antigen or against ICAM-1 donor cell antigen, for preventing the activation of pro-inflammatory mononuclear cells. The antibody has for effect to hinder the binding between ICAM-1 and LFA-1, and this hindrance resulted in a remarkable survival rate of transplanted cells after the first critical 3 to 7 days following transplantation.

Although the use of anti-LFA-1 or anti-ICAM-1 achieved an efficient decrease in the early cell mortality rate, there is still room for improving the success of survival, and for improving the overall success of cell transplantation.

STATEMENT OF THE INVENTION

The present invention covers significant improvements for each event involved in the transplantation success or graft survival. These improvements, separately or combined with each other, greatly ameliorate the recovery of a tissue towards a normal function.

The first object of the invention is to reduce the early death of the transplanted cells. As alternatives to the use of anti-LFA-1 or anti-ICAM-1 antibodies, other anti-inflammatory agents are proposed, alone or in combination. One or more of these anti-inflammatory agents may be combined with the anti-LFA-1 or anti-ICAM-1 antibody, to prevent the early death of transplanted cells. The anti-inflammatory agent may be one or more agents selected from the group consisting of TGF-beta1, ligand(s) against

CD18, CD31, CD50, E-selectin and P-selectin. In a specific embodiment, the anti-inflammatory agent is TGF-beta1.

The anti-inflammatory agent may also be an inhibitor of synthesis of oligosaccharides, such as Castanospermine.

It may alternatively be a glucosidase capable of removing oligosaccharides from glycoproteins.

All the above classes of anti-inflammatory agents have for effect to hinder the binding of pro-inflammatory cells to transplanted cells, or to inhibit the recruitment of pro-inflammatory cells on the transplantation site. Their effect results in the inhibition of pro-inflammatory cell action. The end effect is that pro-inflammatory cells either cannot activate themselves or at least cannot kill the transplanted cells.

The step of early death of transplanted cells being overcome, the next one to be addressed is the diffusion of the donor cells into the recipient tissue. To that effect, this inhibition of formation of glycoproteins is useful as well as the promotion of the formation and secretion of metalloproteases are useful. The combinations of the effect of an anti-inflammatory and of a metalloprotease treatment constitute a second object of the present invention. Metalloprotease treatment can be achieved by treating the donor cells with inducers like phorbol esters or concanavalin A, or directly co-injecting the donor cells with metalloproteases, or injecting the host tissue with metalloproteases, or else by genetically modifying the donor cells to produce the metalloproteases.

Combining treatment of donor cells with metalloproteases with the early anti-inflammatory treatment provides for a maximum of chances that donor cells colonize the recipient tissue. Such combined treatments permit an optimal spreading of the transplanted cells, which can fuse with the recipient tissue cells and are ready to restore a functional protein and tissue function. Metalloproteases promote the fusion of donor with recipient cells. Inhibiting the synthesis of oligosaccharides or detaching the oligosaccharide part from glycoproteins may also promote the diffusion of the transplanted cells by preventing their immediate fusion with recipient tissue cells. As well, avoiding the formation or degrading glycoproteins renders less possible the functional contacts of the donor cells with the recipient cells and connective tissue, and it further prevents adhesion to pro-inflammatory cells and immune system cells.

A third object of the present invention relates to the combination of one of the first two treatments, or both, with to a third step for increasing to any possible extend the number of cells available for transplantation: the ex vivo growth of donor cells. In the international patent publication WO 96/28541, I proposed to grow myoblasts *in vitro* in the presence of growth factors such as b-FGF. Growing the donor cells in the presence of suitable growth factors increases the success of transplantation perhaps by increasing survival and diffusion of the cells. In the case of myoblasts, the treatment

of donor cells with b-FGF increased by 4-fold the number of functional cells resulting from their fusion with the recipient muscle cells. This treatment was as efficient as another pre-treatment which consists of pre-conditioning the recipient tissue with a toxin or an irradiation. *In lieu* of encouraging the *in vivo* proliferation of transplanted cells over the recipient tissue by damaging the latter, the donor cells are stimulated to proliferate by an ex vivo treatment, and these cells are transplanted with no damaging pretreatment of the recipient tissue, , such as irradiation and notexin. Of course, this does not exclude that both a trophic or growth factor and pretreatment with a myotoxic agent can be performed, which may improve the results.

Another way to promote cell proliferation has been tried with success, that is conditionally immortalized donor cells. One way of doing this is to modify the donor cells to express a thermosensitive mutant of SV40 large T antigen. Proliferation occurs at 33°C. Proliferation is stopped and differentiation occurs after shifting at 37°C. These modified donor cells should not be tumorigenic upon transplantation. An additional precaution may be to remove the T antigen before transplanting the cells. This proliferation step may be done in the presence of growth factors. The cell proliferation may also be increased by introducing the telomerase gene or the oncogene c-myc gene, as well as other oncogenes. These oncogenes should, however, be removed before the cell transplantation.

It is a fourth object of the invention to solve the problem of availability of donor cells expressing a functional protein. To avoid the use of immunosuppressive therapy, which have severe side effects, the best thing to do is to match the donor with the recipient cells for histocompatibility. It is almost impossible to achieve the perfect MHC (major histocompatibility complex) match and even with such a perfect MHC match immune problems due to minor antigens will occur. Two solutions are readily apparent: on one hand, using a specific immunotherapy or the development of tolerance on the other hand, using autologous donor cells. Transplanting autologous cells having the same vocation or function appears for the moment the solution of choice, although the source of cells may be limited. In the specific case of patients suffering of muscular dystrophy, a first problem is the availability of myoblasts, because the muscles of these patients are atrophied and/or occupied by a larger than normal connective tissue and, moreover, the remaining myoblasts are senescent or near senescence. The present invention relates to a new way by which other cell types such as mesenchymatous cells and fibroblasts can be converted into myoblasts, by inserting one or more members of a myogenic gene family. The myoD family comprises at least the following six members: myoD, myogenin, herculin, myf-5, myf-6 and MRF-4. Fibroblasts coming from a foreign donor or from the patient himself can be grown *ex vivo* and modified by the insertion of 1) a myogenic gene and of 2) a gene expressing a functional protein corresponding to the protein defective in the diseased subject. If the patient to be

treated does not have any inherited defect in the protein of interest, the promoter of the gene encoding this protein may also be derepressed or replaced by an active one.

Having said previously that myoblasts transplantation may be used to treat muscular dystrophy, heart insufficiency, nanism, hemophilia and Parkinson disease, it will be readily appreciated that the transformation of mesenchymatous stem cells or of fibroblasts into myoblasts opens an interesting way by which a given source of tissue may be used *in lieu* of myoblasts for restoring a functional protein.

A fifth object of the invention is an alternative way by which a functional dystrophin-like protein may be restored. Instead of transplanting cells expressing a functional dystrophin, the promoter of a dystrophin-related protein, utrophin, may be mutated. The result of this mutation would be that utrophin is no longer confined to the synaptic junction, but is expressed through the whole muscle. Utrophin being closely related to dystrophin, a functional tissue is restored. This represents an alternative way by which dystrophin function may be restored in a recipient tissue. Heterologous and autologous transplantation may be therefore performed with genetically modified recipient cells. It is therefore possible that fibroblasts, for example, are converted into myoblasts by inserting myogenic genes, and by inserting a functional dystrophin gene or mutating the utrophin promoter.

Overall, the cells to be transplanted may be modified to express specific proteins. Some proteins are of course the proteins to restore for recovering a normal tissue function. Ischemia produced following occlusion of blood vessels triggers the death of many cells. In the heart, such infarctus leads to the death of many cardiomyocytes. The loss of these cells leads to atrophy of the cardiac tissue and ventricle dilatation. Transplantation of skeletal myoblasts in the heart could prevent this atrophy of the cardiac muscle as it does for the skeletal muscles. Although these skeletal myoblasts may not differentiate into cardiomyocytes, they may nevertheless form a more elastic and contractile tissue. Moreover, if the transplanted myoblasts are genetically modified to express connexin, this may permit electrical coupling between them and the cardiomyocytes and allow synchronous contraction with the rest of the heart compartment. Some other proteins include trophic gene products, anti-inflammatory gene products, metalloproteases, glucosidases or inhibitor of synthesis of oligosaccharides, growth factors, hormones, coagulation factors or MHC proteins or any combination thereof.

In the case wherein the transplanted cells are myoblasts, the genetic modifications may include myogenic genes introduced in another cell type, which transforms the latter into myoblasts.

Finally, in the case wherein the cells to be transplanted are not of an autologous source or cannot be made histocompatible with the recipient subject, the genetic modifications can also include the expression of molecules involved in specific

immunotherapy (i.e., development of specific tolerance). Specific immunotherapy is the best way to avoid severe side effects and to encourage tolerance to the graft by the humoral and cellular immune system. An increasing body of literature exists on the identity of cell surface determinants (stimulatory or co-stimulatory proteins and of receptors which are responsible for immune reactions). Once a specific set of antigens or receptors is identified as taking part to the graft rejection mechanisms, it is feasible to design suitable protein ligands which interfere with these cell surface determinants or their receptors. Such ligands should prevent the recruitment of blood cells, inhibit their binding with the transplanted cells and the activation of blood cells which normally kills the transplanted cells. The donor cells may therefore be modified to express such proteic ligands, which provides for the necessary specific immunotherapy. Of course, such a specific immunotherapy (tolerance) is a gold standard which will ideally replace the non-specific immunotherapy which has severe side effects. An indication that a specific immunotherapy will work properly is already provided when considering the remarkable success achieved with an anti-LFA-1 antibody.

Not all the genetic modifications need to be made in the same cells. For example, a donor cell which expresses a ligand for binding leukocytes (to prevent their recruitment, binding or activation) does not necessarily have to express other foreign genes. The cells expressing an anti-LFA-1 antibody, for example, may be co-transplanted with other cells expressing a metalloproteases, and with a third cell type expressing a functional tissue protein. Of course, cells may also be genetically manipulated to express more than one foreign protein. A typical example of this would be fibroblasts transformed with both a myogenic gene and a functional tissue protein like dystrophin, to make a functional myoblast. Therefore, a mixture of different recombinant cells may be transplanted in a recipient tissue, each having a specific vocation, e.g., breaking down the connective tissue, keeping the pro-inflammatory cells away or preventing their activation, keeping the whole recipient subject tolerant to the graft, replacing a defective protein or expressing any protein of interest.

DETAILED DESCRIPTION OF THE INVENTION:

The present invention provides for improved methods to restore missing functional molecules in affected individuals, which restoration is greatly improved by solving each above pointed out problems. In dystrophic patients, transplantation of myoblasts (e.g., restoration of dystrophin) or the transplantation of non myogenic cells (e.g., restoration of merosine) will achieve such restoration. The invention is based on successful demonstrated restoration of these molecules (Vilquin et al., 1994 a,b, 1995a,b; Huard et al. 1991 a,b; 1992 a, b; 1993, 1994 a,b; Tremblay et al. 1991 b; 1993 a,b; Roy et al. 1993; Kinoshita et al. 1994 a, b,c; Asselin et al. 1994; 1995).

The claimed methods have been demonstrated in mice affected of various hereditary diseases, in monkeys and in Duchenne Muscular Dystrophy patients (see references above and Roy et al. 1991; Tremblay et al. 1991a; Labrecque et al. 1992; Satoh et al. 1992, 1993; Albert and Tremblay 1992; Meola et al. 1993; Sansone et al. 1993; Belles-Isles et al. 1993; Vilquin et al. 1995).

Dystrophic muscle fibers degenerate because of the lack of a normal gene product. Cell transplantation can restore this missing gene product either in the case of myoblasts by forming new muscle fibers or forming hybrid muscle cells (i.e. by fusing with host myoblasts or muscle fibers) containing the missing gene product (e.g., dystrophin, sarcoglycans or dystroglycans). In the case of transplantation of non-myogenic cells, the missing gene product (e.g., merosin) may be secreted in the extracellular matrix. Extracellular proteins may also be secreted by any other type of genetically modified cells either injected directly in the muscle or invading the muscles following their injection in the blood. Among the cells that easily invade a muscle where there are ongoing cycles of damage and regeneration, are neutrophils and macrophages. Thus genetically modified macrophages and neutrophils (or their precursor stem cells) could secrete extracellular proteins, such as merosin, in the muscles. This approach would have the additional advantage that merosin could also be secreted around nerves or within any other tissues having damages due to the absence of this extracellular protein. The same approach could also be used for any matrix protein.

The myogenic or non myogenic cells to be transplanted can be MHC compatible or incompatible with the host. In both of these cases and in cases where a new gene product is introduced by the transplanted cells, adequate immunosuppression will be required. This immunosuppression may be assured with pharmacological agents such as cyclosporine-A, rapamycin or FK506 (Kinoshita et al. 1994a, b; Vilquin et al. 1994, 1995a, b). The immunosuppression may also be induced by monoclonal antibodies against various lymphocytes or antigen presenting cell determinants or by genes encoding the antibodies or antibody fragments or recombinants proteins which are the receptors or part of a receptor for the binding proteins. A suitable regimen therapy using a combination of CTLA4-Ig combined with an anti-CD4 mAb or combination of anti-CD4, anti-CD8 and anti-LFA-1 mAbs were both capable of increasing the number of positive cells temporarily in MHC-incompatible subjects. It is suggested that the regimen therapy should be carefully monitored in such a way that the dose is adjusted to still permit the formation of suppressor T-cells which may be responsible for the development of tolerance. These immunosuppressive agents may maintain long-term graft survival and induce tolerance to the myoblasts and muscle fibers of donor origin.

The immunosuppressive treatment may therefore be induced by non-specific suppressive agents or by more specific ones.

The used of mAbs may eventually lead to permanent tolerance for the new antigens introduced by the cell transplantation procedure (see review by Waldman and Cobbold 1993).

The myogenic and the non myogenic cells may be grown and cloned using several different methods. Several examples of such methods have been described by the inventor (for human myoblast culture technique see Tremblay et al. 1991a, b; 1993a, b; Huard et al. 1992a, b). Experts in tissue culture may, however, modify these methods to reach the same purpose. The cloning of cells may also be facilitated by introducing in the founder cell a gene to induce a conditional immortalization. The use of cloned cells may also prevent undesired effects produced by other cell types present in primary muscle cell cultures.

A mouse cell line MB3 has been produced which contains the heat sensitive SV40 T antigen under control of the IFN-gamma inducible H-2 Kb promoter. The MB3 cells were transfected using the retrovirus LNPOZ (kind gift of Dr. D. Miller, Fred Hutchinson, Cancer Research Center, Seattle, WA) which contains the LacZ reporter gene and a neomycin resistance gene driven by retroviral LTR (Adam et al. 1991). The clone 3LN was obtained by further selection for resistance to geneticine (200 microg/ml) and beta-Gal expression, and expanded for use in transplantation experiments. The survival of the beta-Gal labeled cells was assessed by dosing the beta-Gal activity (Sambrook et al. 1989) in homogenates of control muscles (obtained 3 hours after the transplantation) and in muscle homogenates obtained three days after the transplantation.

The method of increasing the proliferation of the transplanted cells by introducing a gamma-IFN inducible SV40 T antigen, c-myc gene or the telomerase gene may be subject to variation, as will be apparent to the skilled artisan, to reach the same goal. Animal models are applicable to other mammals including humans. To evaluate how a non-defective protein is produced in a recipient individual, a PCR reaction may be made on a biopsy. The messenger RNAs so amplified may have a differential restriction enzyme pattern on an electrophoretic gel. This has been shown to be the case for the dystrophin cDNA which is different in *mdx* mice and in C57BL/10SnJ donor mice, (Asselin et al. (1994 and 1995)), as well as for TGFbeta (see below). Furthermore, the fate of transplanted myoblasts may be monitored by way of fluorescent latex microspheres labeled myoblasts (Satoh et al.,1993).

The amount of cells to be injected may depend of the type of disease to be treated, of the severity and stage of the disease and of the type of cells to be injected for the treatment. In the case of myoblasts it may be necessary to inject these cells throughout the skeletal or cardiac muscle because these cells do not diffuse very well

in the muscle. The distribution of the myoblasts throughout the skeletal or cardiac muscles may be facilitated by developing a device controlled by a robot. This robotic device may receive from an imaging system (e.g., magnetic scanner, echography or low intensity X-rays) the information describing the exact muscle shape and size, and the position of bones, nerves and blood vessels. The robot will use this information to plan injection trajectories avoiding the major nerves and blood vessels and thus avoiding damaging them.

The cells required to treat the muscle may also be inserted by some other routes. For example, the cells may also be distributed in the skeletal and cardiac muscles by injecting them in blood vessels of the patient and genetically modify them so that they not only attach to blood vessel cells but also cross the blood vessel and migrate in the tissue. The expression of adhesion molecules or metalloproteinases may be increased by stimulating the cells with cytokines (e.g., proinflammatory cytokine, IL-1). The secretion of these transplanted cells may also diffuse in the muscles and eventually in the blood. This could be the case for merosin or for factor VIII for example.

The solution to inject the cells may range from simple sterile saline isotonic solution, to sophisticated culture mediums containing growth factors such as bFGF) and/or metalloproteases to facilitate the cell survival and migration. The injection solution may also contains pharmacological agents and monoclonal antibodies to prevent death of the injected cells. These solutions will have to be prepare using Good Laboratory Practice. Since one of the principal objects of this invention is to enhance the early survival rate of transplanted cells, monoclonal antibodies at least comprise an anti-LFA-I antibody (if administered directly to the patient) or an anti-CAM-I antibody fragment, alone or in combination with any anti-inflammatory agents.

The claimed invention can be clarified by examples of experimental treatments in animals and human patients. The experiments as well as the appended figures are examples and should not be used to limit the scope of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: TGF-β1 gene expression increased myoblast transplantation survival. Percentage of myoblast death 3 days after transplantation in *Tibialis anterior* muscle of CD1 mice. Transplantations were performed with CMV LacZ myoblasts (Group 1: 8 mice), CMV LacZ myoblasts infected with TGF-β1 retrovirus (Group 2: 10 mice) and CMV LacZ myoblasts co-transplanted with a TGF-β1 G8 myoblast clone (Group 3: 10 mice). Each value is the mean ± SEM. * : significantly different from group control (Group 1) at $P<0.05$ (Student's *t*-test).

Figure 2: Products of RT-PCR amplification with TGF-β1 primers of total RNA extracts from untransplanted TA muscle (lane b), TA muscle transplanted with CMV

LacZ myoblasts (lane c), TA muscle transplanted with infected TGF-β1 myoblasts (lane e) afetr 3 days. The amplification products were digested with restriction enzyme *Mae*III. Lane d: TA muscle transplanted with CMV LacZ myoblasts. Lane f: TA muscle transplanted with infected TGF-β1 myoblasts. Lane a: molecular weight standard.

Figure 3: Decrease of neutrophil in the blood after transplantations with myoblasts expressing TGF-β1. Myeloperoxidase activity was determined in blood in control CD1 mice which were not transplanted with any myoblast (Control: 4 mice) or in CD1 mice 3 days after transplantation of myoblasts in *Tibialis anterior* muscle (Group 1 to 3). Transplantations were performed with only normal CMV LacZ myoblasts (Group 1: 4 mice), CMV LacZ myoblasts infected with a TGF-β1 retrovirus (Group 2: 6 mice) and CMV LacZ myoblasts co-transplanted with TGF-β1 G8 myoblast clone (Group 3: 6 mice). Each value is the mean ± SEM. †: significantly different from Control; *: significantly different from Group 1 at $P<0.05$ (Student's *t* -test).

Figure 4: Infection of myoblasts with TGF-β1 retrovirus decreased the release of oxidant by neutrophils. CMV LacZ myoblasts (Group 1), CMV LacZ myoblasts infected with TGF-β1 retrovirus (Group 2) and CMV LacZ myoblasts mixed with a TGF-β1 G8 myoblast clone (Group 3) were incubated with interleukin-1β (IL-1β), loaded with C-DCF and mixed for one hour with neutrophils activated with zymosan activated serum (ZAS). The fluorescence intensity of the C-DCF was measured at different intervals after the addition of neutrophils. Each value is the mean ± SEM of 6 distinct experiments. *: significantly different from Group 1 at $P<0.05$ (Student's *t* -test).

Figure 5: Effect of castanospermine (1,5, and 10 mM) on myoblast oxidation by PMNs. The positive control is myoblasts exposed to PMNs activated with Zymogen Activated Serum.

Figure 6: *In vivo* effect of castanospermine on myoblast β-gal marker activity. Myoblasts were treated with castanospermine during 24 hours prior to transplantation. Treated animals were sacrificed after 3 days. Control animals consist of animals sacrificed 1 hour after transplantation without pretreatment with castanospermine.

Figure 7: shows the number of muscle fibers positive for X-Gal counted after an injection of 500 000 donor's cells in one site of the *tibialis anterior* of recipient mice. Imm 7 neo: expresses neomycin. Imm 7 Matrilysine: expresses neomycin and matrilysine. Tn I βgal: untreated transgenic mouse myoblasts expressing β-Gal. Tn I βGal + TPA: transgenic mouse myoblasts / β-Gal treated with phorbol ester.

Figure 8: Modification of dermal fibroblasts with MyoD1. Expression of muscle markers, i.e. desmin (a) and MyoD1 (b) is shown by immunostaining in cultures of dermal fibroblasts infected with a retroviral vector expressing MyoD1. *in vitro* fusion and β-gal expression in MyoD1-infected TnI LacZ fibroblasts (c). Dystrophin immunofluorescence in fused genetically modified fibroblasts (d). Fusion and β-gal

expression is not detected in uninfected fibroblasts (e). β-gal expression in mdx mouse muscles 1 month post-transplantation of control (f) or MyoD1-expressing fibroblasts (g) and TnI LacZ myoblasts (h). No dystrophin was detected by immunofluorescence (i) on the β-gal + fibers (same fibers as in g) obtained by the fusion of the MyoD1-infected TnI LacZ fibroblasts. Hematoxylin-eosin coloration on transplanted muscle revealed the presence of abundant connective tissue (j). (a, b, h: 100x; c, d, e, f, g, 200x; i, 400x; j, 25x).

EXAMPLES

EXAMPLE 1

Anti-inflammatory effect of TGF-beta1 in myoblast transplantation

Myoblast transplantation is one of potential treatments of different muscular dystrophies. Myoblast transplantation in mice resulted in the expression of a reporter gene or of a lacking protein (i.e., dystrophin) in more than 90% of host fibers. These myoblast transplantations were performed either in muscle of histocompatible or immunosuppressed mice to avoid the specific immune response. Despite the control or the prevention of the specific immune response, there was an inflammatory reaction which occurred during the first 3 days after myoblast injection. Our group has recently presented evidences that this inflammatory reaction was responsible for the rapid myoblast death of up to 99% of the myoblasts observed by many laboratories. Previous experiments of our group have indeed shown that neutrophils and natural killer cells were infiltrating the muscle within a few hours after transplantation were directly implicated in the cytotoxicity against myoblasts (Merly F, et al. (1998) Transplantation 65(6):793-799). In mice, this cell death seems to be compensated by an intense proliferation of remaining myoblasts and does not affect the success of the transplantation, but the rapid cell death due to inflammation is perhaps responsible for the limited success of transplantation in monkeys or in clinical trial with DMD patients specially when a low number of myoblasts where transplanted.

Myoblast mortality has been previously reduced by treating the host with a monoclonal antibody against LFA-1 (CD11a/CD18). This protein is expressed on polymorphonuclear cells, on natural killer cells and lymphocytes and interacts with ICAM-1 present on myoblasts. Results of our group suggested that the fixation of the antibody on LFA-1 prevented the interaction of neutrophils with myoblasts and thus limited the cytotoxic activity against injected cells. This very efficient method in mice will require high dose of mAb if it would be applied to DMD patients. It is therefore necessary to find a new medication to prevent inflammatory reaction before the next clinical trials. A first solution is to genetically engineered the myoblasts to express and secrete anti-LFA-1 antibodies. A second solution is to find other cytokines which alone or in combination will achieve an efficient anti-inflammatory effect.

Transforming Growth Factor-beta 1 (TGF-beta1) is a 25 Kd cytokine produced

by many cells including platelets, bone cells, macrophages and fibroblasts. This protein is a multifunctional cytokine that plays central roles in embryonic development, wound healing and particularly immunoregulation. Different studies have shown that TGF-beta1 has important anti-inflammatory properties. It decreases the expression of IL-1beta and of its receptor and increases synthesis and secretion of IL-1Ra, a natural receptor antagonist of the IL-1 receptor. TGF-beta1 has been shown to inhibit the transmigration of neutrophils through activated endothelium and the secretion of cytolytic product such as nitric oxide or granzyme B by polymorphonuclear and natural killer cells. In some cases, TGF-beta1 promotes opposite activities: applied locally, it promotes, *in vivo*, leukocyte adhesion, infiltration and activation. It has been shown to stimulate *in vitro* degranulation and oxidant release by adherent human neutrophils.

Previous studies suggested that TGF-beta1 could be an important therapeutic adjunct in the prevention of transplantation rejection. Indeed, systemic injection of recombinant TGF-beta1 or pre-treatment of donor transplant with TGF-beta1 prolonged the survival of heart and islet allo- or xeno- graft in mice.

The aim of this study is the prevention of inflammatory reaction after myoblast transplantation. We examined *in vitro* and *in vivo* the anti-inflammatory effects of recombinant human TGF-beta1 in mice. The DFG retroviral vector containing human TGF-beta1 gene under control of the retroviral LTR was used to infect myoblasts before their transplantation. The results show that expression of TGF-beta1 by injected cells reduced significantly their death by preventing the recruitment of PMNs and by decreasing the cytotoxic activity of neutrophils against myoblasts.

MATERIAL AND METHODS

Myoblast culture

CMV LacZ transgenic mouse line (line 9) (generous gift of Dr K. E. Hasting, McGill University, Montreal, P.Q., Canada) was produced by pronuclear injection of a 5 Kb XhoI/SpeI fragment (Cayla, Toulouse, France) including the human cytomegalovirus (CMV, Towne strain) immediate early enhancer/promoter region (-511 to +97 relative to IE1 transcription start site) driving expression of E. coli LacZ beta-gal. The transgene was maintained on a CD1 mouse background. Primary myoblast cultures were prepared from hemizygous transgenic 2 days old mice. After enzymatic dissociation with collagenase 0.2% and trypsin 0.25%, myoblasts were cultured in M199 medium at 37C for two days.

G8 myoblasts, clone CRL 1447, initially isolated from hind limb of fetal Swiss Webster mice (ATCC, Rockville, MA, USA) were grown in culture in Dulbecco's modified Eagle's medium with 10% fetal calf serum (DMEM-FCS).

Retroviral vector and *in vitro* infection

Retroviral DFG-TGF-beta1 vector encodes for recombinant human Transforming Growth Factor-beta1 under the control of the retroviral LTR. The human TGF-beta1 gene was inserted into the MFG vector at the NcoI and BamHI cloning sites. An IRES-Neo-cassette was inserted 3' to TGF-beta1, allowing the expression of two genes from a polycistronic message. Virus was prepared by transfection of BOSC23 cells.

This retroviral vector was directly added to myoblast culture (G8 or CMV LacZ) with polybrene (8 microg/mL) overnight. CMV LacZ myoblasts were infected 3 times. G8 myoblasts were infected only once but after 4 days in DMEM-FCS in presence of 400 microg/mL of G418, they were cloned by limited dilution in 96 well plates. The resulting clones of G8 and primary CMV LacZ myoblasts cultures infected with this vector were grown and assayed for TGF-beta1 production.

TGF-beta1 assay

ELISA :

TGF-beta1 was assayed by ELISA. A 96-well flat-bottom plate was coated with human TGF-beta1 (Genzyme) for the standard curve, with culture supernatants or with mouse serum and incubated 2 hours at 37C. After 3 washes with PBS containing 0,5% Tween 20, non specific sites were blocked by PBS-milk 5% overnight at 4°C. Plates were again washed and incubated with 0.1 microg of a rabbit IgG against TGF-beta1 (R&D System inc.) for 2 hours at 37C. This antibody reacts with human TGF-beta1 but also crossreacts with mouse TGF-beta1, TGF-beta2 and TGF-beta3. Plates were washed and incubated with a goat anti-rabbit IgG coupled to biotin (1/1000) 1 hour at 37C. After 3 washes, plates were incubated with streptavidin-horse radish peroxidase (HRP) (1/500) for 1 hour at 37C. Plates were washed and 200 microL of OPD (o-phenylene-diamine-2HCl) (Kit OPD, Abbott Diagnostic) were added to each well. Reaction was developed for 15 min. at room temperature and stopped with H_2SO_4 (2N). Plate ODs were measured at 492 nm. Results were expressed as means +/- SEM.

RT-PCR :

RNA isolation was performed using Trizol reagent (Gibco BRL, Burlington, Ontario, Canada). Muscles were homogenized in 1 mL of Trizol and 0.2 mL of chloroform was added to the homogenate. RNA fraction was isolated by centrifugation (12000 g, 5 min., 4°C). After precipitation with isopropyl alcohol (0.5 mL), RNA was washed in ethanol 75% and redissolved in RNAse free water. RNA was dosed at 280/260 nm, using a spectrophotometer.

RT-PCR was carry out using the antisense primer 5'-GCGCCCGGGTTATGCT GGTTGTA-3' and the sense primer 5'-CGGAGTTGTGCGGCAGTGGTTGA-3' producing a 449 bp sequence of mouse or human TGF-beta1. For reverse transcription, 50 ng of antisense primer were added to 200 ng of total RNA sample and

completed with RNAse free water. RNA was denatured at 60°C for 5 min. and cooled for a few seconds on ice. Complementary DNA was synthesized by adding 4 microL buffer 5X (Gibco BRL), 2.5 microL of dNTPs (10 mM) (Pharmacia, Baie d'Urfé, Québec, Canada), 2 µL of 100 mM DTT (Gibco BRL), 0.5 microL of RNAse inhibitor (Boehringer Mannheim, Laval, P.Q., Canada) and 1 microL of reverse transcriptase (Gibco BRL) and incubating for 1h at 42°C.

The cDNAs were amplified in a 100 microL reaction volume containing sense and antisense primers (400 ng), 10 microL of PCR buffer 10X and 0.5 microL of The polymerase (Boehringer Mannheim). The PCR amplification was done at the following temperature : 94°C for denaturation, 65°C (30 cycles) for the annealing and 72°C for the extension.

PCR products were digested for 5 h at 55°C with the restriction enzyme MaeIII (3 U/microg DNA) in a total volume of 20 microL containing 1 microL BSA (2 mg/mL), 2 microL buffer 10X completed to 20 microL with water. Only one MaeIII restriction site was present in the 449 bp amplicon of mouse TGF-beta1. Thus the digestion with this enzyme produced two fragments (72 and 377 bp). Two MaeIII restriction sites were present in the amplified segment of human TGF-beta1. Therefore after complete digestion of human 449 bp amplicon with MaeIII, 3 fragments were obtained (72, 60 and 317 bp).

Myoblast transplantation

Myoblast transplantations were performed in Tibialis anterior (TA) muscle of CD1 mice (Jackson Laboratories, Bar Harbor, ME, USA). The skin of hind limb was cut to expose the TA muscle. Roughly 106 CMV LacZ or TGF-beta1-CMV LacZ myoblasts were injected using a micropipette (Drummond Co., Broomal, PA, USA) at different sites in each muscle. For cotransplantation of CMV LacZ myoblasts and G8-TGF-beta1, 10^6 cells of each type were injected. The skin was sutured with 6.0 silk (Johnson & Johnson, Peterborough, Ont., Can).

Myeloperoxidase (MPO) assay

Myeloperoxidase enzyme activity in muscle was dosed by a modified method described by Bradley et al. Briefly, blood was diluted (1/2) in potassium phosphate buffer (50 mM, pH 6.0). After 30 sec of sonication, the solution was frozen in liquid nitrogen, thawed 3 times and centrifuged 5 min. at 12500 rpm. A solution of o-dianisidine dihydrochloride (0.0167%) and 0.0005% H_2O_2 in KH_2PO_4 (50 mM, pH 6.0) was added to the blood supernatant. Changes in absorbance at 460 nm were monitored with a spectrophotometer every 30 sec for 5 min.

Beta-Galactosidase (beta-gal) assay

Muscles were homogenized in 800 microL of 0.25 M Tris-HCl pH 7.8. After centrifugation (5 min., 6500 rpm), supernatants were kept at -20°C until beta-galactosidase assay. Supernatant (100 microL) was mixed with 3 microL of a solution

containing 0.1 M $MgCl_2$, 4.5 M beta-mercaptoethanol, 66 microL of o-nitrophenyl-β -D-galactopyranoside (4 mg/mL) and 131 microL of sodium phosphate (0.1 M, pH 7.5). Samples were incubated 1.5 hour at 37°C. The reaction was stopped by adding 150 microL of 1 M Na_2CO_3. The optical density was read at 420 nm on spectrophotometer. Standard curve was made with a solution of beta-galactosidase extracted from *Escherishia coli* (Sigma, St-Louis, MO, USA).

Neutrophil isolation and *in vitro* cytotoxicity assay

Casein 8% (w/v) was injected intraperitoneally (IP) in CD1 mice. Six hours after injection, mice were injected IP with HBSS and massaged gently. Neutrophils were removed from the peritoneal cavity with a plastic pipet.

Myoblasts (10 000 per well) were plated in 96 well plates and grown for 1 day. For some wells, myoblasts were incubated 3 hours at 37°C with recombinant mouse interleukine-1beta (IL-1beta) (4 U/mL) (Genzyme diagnostics, Cambridge, MA, USA). C-DCF (6-carboxy-2',7' dichlorodihydrofluoroscein diacetate diacetoxymethyl-ester) (Molecular Probes inc., Eugene, OR, USA) was added to cells for 20 min. at 37°C. After 3 washes with HBSS, myoblasts were incubated with neutrophils (50 neutrophils per myoblast) in presence or absence of 1% zymosan activated serum (ZAS). Oxidation of myoblasts by neutrophils was monitored with a fluorometer (excitation 485 nm, emission 530 nm) for 60 min. Results were expressed as DOD +/- S.E.M.

Immunohistochemistry

After perfusion with NaCl 0.9%, muscles were removed and placed in PBS with 30% sucrose overnight at 4°C. Muscles were then embedded in OCT (Shandon, Pittsburgh, PA, USA) and frozen in liquid nitrogen. Sections of 30 mm were cut at -22°Cwith cryostat microtome and picked up on gelatin coated slides.

X-Gal staining:

Cross sections were fixed in 1% of glutaraldehyde for 3 min. After 3 washes, slides were incubated overnight with X-Gal solution and mounted in PBS-glycerol.

MAC-1 immunohistochemistry:

Cross sections were washed with TBS-casein 3 times and non specific sites were blocked with 10% horse serum for 15 min. Slides were incubated with rabbit anti-MAC-1 IgG for 1 hour at room temperature. After fixation in methanol with 0,03% H_2O_2, primary antibody fixation was revealed by using anti-rabbit IgG biotinylated and Streptavidin HRP. The HRP activity was revealed with diaminobenzidine. Slides were mounted in PBS-glycerol.

RESULTS

Three days after infection with the retroviral DFG-TGF-β1 vector, no modification of the morphology or death was observed in CMV myoblast cultures. Similar results were obtained after selection by G418 and cloning of TGF-beta1 G8 cells. TGF-beta1 production by myoblasts was assessed by ELISA. Culture

supernatants were dosed for both types of cells (CMV LacZ and G8 myoblasts). TGF-beta1 production was similar for G8 and CMV LacZ myoblasts infected by DFG-TGF-beta1 retrovirus (respectively 171 ± 30 and 191 ± 3 ng/day/10^6 cells).

TA muscles of CD1 mice were transplanted either with 1) CMV LacZ myoblasts (10^6 cells) alone or 2) with CMV LacZ myoblasts co-transplanted with TGF-β1 G8 cells (10^6 cells) or 3) with DFG-TGFβ-1 infected CMV LacZ myoblasts (10^6 cells). Muscles were removed 1 hour or 3 days following transplantation. β-galactosidase activity was assayed for each muscle. The percentage of death of injected CMV LacZ myoblasts was defined as the difference between β-gal activity at 1 hour and 3 days divided by the β-gal activity at 1 hour multiplied by 100 (Figure 1). As previously described (11), almost 70% of control myoblasts died during the 3 days following transplantation. After infection with retroviral DFG-TGFβ-1, CMV LacZ myoblast mortality was significantly reduced, since only 46.3 ± 4.2% of the myoblasts were dead after 3 days. The same reduction of myoblast death was observed following cotransplantation of CMV LacZ and TGFβ-1 G8 myoblasts, the percentage of death being 46.2 ± 5.9%.

Three days after myoblast transplantation, TGFβ-1 expression was quantified in TA muscles by RT-PCR. As shown in figure 2, control transplantation of normal myoblasts in TA muscles induced only a slight activation of mouse TGFβ-1 expression (lane c). Digestion with the restriction enzyme MaeIII produced a 377 bp fragment (lane d) characteristic of mouse TGFβ-1. In contrast, transplantation of myoblast expressing human TGFβ-1 increased markedly the expression of TGFβ-1 (lane c). Following the transplantation of these infected myoblasts, digestion of the RT-PCR product with MaeIII enzyme produced both the 317 and the 377 bp fragments (lane f). This clearly indicated the presence of two components: an endogenous mouse TGFβ-1 and an exogenous recombinant human TGFβ-1.

The measurement of TGFβ-1 by ELISA in the serum did not reveal any significant difference in the circulating TGFβ-1 levels in mice transplanted with normal or infected CMV LacZ myoblasts (with respectively 653 ± 112 ng/mL and 684 ± 45 ng/mL).

Myoblast transplantation in TA muscles led to an inflammatory reaction during the first 3 days following cell injection. This non specific response was quantified by MPO assay in blood (specific enzyme of neutrophils and macrophages) (Figure 3) and by immunohistochemistry on muscle cross sections using anti-MAC-1 monoclonal antibody specific of polymorphonuclears and macrophages (not shown).

MPO activity in blood characterized the number of circulating neutrophils. In untransplanted mice, the MPO activity was at basal level with 9.4 ± 2.3 10^{-3} ΔOD/min. After transplantation of CMV LacZ myoblasts in TA muscles of CD1 mice, MPO activity in blood increased markedly up to 47.5 ± 6.9 10^{-3} ΔOD/min at 3 days. This increase was related to recruitment of neutrophils following transplantation. For mice

transplanted with CMV LacZ infected with the DFG-TGFβ-1 retroviral vector, the increase in MPO activity in blood was lower than for mice transplanted with uninfected myoblasts (27.0 ± 7.5 10^{-3} ΔOD/min.). Similar results were obtained for the cotransplantation of CMV LacZ myoblasts with TGFβ-1 G8 myoblasts (Figure 3).

Observations of cross sections stained by anti-MAC-1 antibody were correlated with MPO activity variation in blood (not shown). TA muscles transplanted with CMV LacZ were infiltrated by many polymorphonuclear cells stained by anti-MAC-1 antibody. Mononuclear cell infiltration was significantly reduced in TA transplanted either with TGFβ-1 CMV LacZ myoblasts or normal CMV LacZ and TGFβ-1 G8 myoblasts.

To determine whether expression of TGFβ-1 by myoblasts can modulate neutrophil cytotoxicity, the release of oxidant, such as oxygen free radicals, by neutrophils was tested *in vitro* by measurement of C-DCF reduction (Figure 5). Experiments were performed by adding neutrophils activated with ZAS (zymosan activated serum) to myoblasts pre-incubated with IL-1 and loaded with C-DCF. In these conditions, infection of the myoblasts with the TGFβ-1 retroviral vector decreased significantly their oxidation by neutrophils in comparison with myoblasts not infected. Similar results were obtained with myoblasts not infected but mixed with G8 myoblasts infected with TGFβ-1 retrovirus.

DISCUSSION

Myoblast transplantation induced during the first week an inflammatory reaction in muscles. The sites of cell injection were indeed infiltrated by many mononuclear cells such as neutrophils, NK cells, macrophages. This non specific response to transplantation was associated with an acute death of injected myoblasts. Myoblast death occurred even following transplantation in immunosuppressed mice or following syngeneic transplantation. Neutrophils and NK cells were directly implicated in myoblast death (Merly et al.)

In the present study, the anti-inflammatory property of TGFβ-1 was used to control myoblast death. Myoblasts were infected with a retroviral vector containing the gene of TGFβ-1. Survival of genetically modified myoblasts was increased at 3 days following transplantation and the same increase survival was obtained when normal myoblasts were co-transplanted with a G8 myoblast clone expressing TGFβ-1. In this experiment, the β-galactosidase assay measured only the survival of CMV LacZ myoblasts and not G8 myoblasts. Thus, TGFβ-1 secreted by the G8 myoblasts protected the non infected CMV LacZ myoblasts.

When applied locally, TGFβ-1 have been shown to promote leukocyte recruitment and infiltration to sites of inflammation. However, when TGFβ-1 was administrated systemically, it had the opposite effect, inhibiting infiltration of leukocytes. In our model of transplantation, we demonstrated that human TGFβ-1 was

only expressed in transplanted muscles and that its level did not increase in serum. This local secretion of human TGFβ-1 at the site of inflammation decreased the polymorphonuclear cell and macrophage infiltration in muscles. The increased expression of human TGFβ-1 in the muscle also resulted in a decreased number of neutrophils in blood (determined by MPO assay). This observation seems initially surprising since the total TGFβ-1 (human ans mouse) was not increased in serum. It is possible that human TGFβ-1 reduces the release of proinflammatory cytokines in the muscle which diffuse in the blood and produce a systemic effect, i.e., reduction of release of neutrophils in the blood from bone marrow. Following the injection of plasmid containing TGFβ-1 gene in a muscle, no infiltration with monocytes or neutrophils was observed. In animal model of gouty attacks, local injection of TGFβ-1 in monosodium urate monohydrate crystal-induced inflammation was also associated with a decrease in leukocyte infiltration. Therefore, the effects of TGFβ-1 are complex, being both pro and anti-inflammatory. In myoblast transplantation, the expression of TGFβ-1 by myoblasts reduced the inflammatory response. *in vitro*, TGFβ-1 inhibits the IL-8 dependent transmigration of neutrophils through activated endothelium and, *in vivo*, it inhibits endothelial cell adhesiveness for neutrophils. Following myoblast transplantation, decrease of leukocyte infiltration by the secretion of TGFβ-1 could be due to an action directly on cell migration and/or indirectly by inhibition of chemoattractant expression in muscle.

We have shown that expression of TGFβ-1 by myoblasts decreased *in vitro* cytotoxicity of neutrophils. Although, a recent study showed that TGFβ-1 stimulated *in vitro* degranulation and oxidant release by neutrophils, it has been previously reported that this cytokine inhibited superoxide release by neutrophils or macrophages. Interaction of ICAM-1 and LFA-1 located on neutrophils induced liberation of free oxygen radicals by neutrophils and thus target cell oxidation. In our *in vitro* experiments, TGFβ-1 decreased myoblast oxidation by neutrophils. Expression of TGFβ-1 could down regulate the expression of ICAM-1 on myoblasts and consecutively decrease the interaction between neutrophils and target cells, as it has been observed with E-selectin. In myoblast transplantation, systemic injection of anti-LFA-1 antibody resulted in a drastic reduction in myoblast death (76% to 18%), after 3 days, without reduction of PMN and macrophage infiltration. In addition, anti-LFA-1 antibody induced a completed inhibition of *in vitro* myoblast oxidation by neutrophils. The effect of anti-LFA-1 was attributed to a direct impairment of the LFA-1/ICAM-1 interaction and thus of neutrophil activation. In the present experiment, TGFβ-1 blocked only partially the inflammatory cell infiltration and the *in vivo* oxidation. It seems, therefore, that TGFβ-1 is less potent at blocking neutrophil activation. This explains why TGFβ-1 did not increase the myoblast survival as much as anti-LFA-1.

IL-1 plays a major role in the initiation and the amplification of the inflammatory

response by activating a cascade of immune cells. It stimulates chemotaxis of leukocytes to the site of inflammation by inducing expression of chemotactic molecules and of adhesion molecules responsible for the migration of neutrophils and monocytes. IL-1 has been also shown to stimulate the production of nitric oxide by neutrophils. Systemic effects of IL-1 include leukocytosis (increased number of circulating mononuclear cells). Our results showed that secretion of TGFβ-1, by transplanted myoblasts in muscle, decreased neutrophil recruitment in muscles and their number in blood. This suggests that reduction by TGFβ-1 of the inflammatory reaction and of the consecutive myoblast death after transplantation could be due to the inhibition of production or action of IL-1 at the transplantation site.

Reduction of inflammatory response following myoblast transplantation allowed an increase of myoblast survival. Use of genetically modified cells could be one of the possible way to release anti-inflammatory products such as TGFβ-1. This procedure permits 1) a local release of anti-inflammatory molecules at the injection site, thus reducing systemic effects, 2) the use of transformed cells co-transplanted with normal myoblasts and 3) the use of cells co-transplanted with gene encoding TGFβ-1 and another protein (structural, immunosuppressive and/or a complementary anti-inflammatory drug or cytokine). These genetically modified cells could eventually in future animal or human experiments be inactivated after the first week by using an inducible promoter, if indicated.

TGFβ-1 secretion by myoblasts decreased inflammatory reaction, but did not abrogated it completely. In many inflammatory models, IL-1 blockade does not also completely prevent inflammation, emphasizing the importance of other inflammatory pathways. TNFα, an other pro-inflammatory cytokine secreted by activated macrophages, has been demonstrated to be an alternative pathway to initiate inflammatory reaction. An important reduction of myoblast death by inflammatory response prevention should be obtained with synergic anti-inflammatory mediators acting on different pathways. This mediator may comprise one or more of the following antagonistic ligands: IL-1, IL-8, E-selectin, P-selectin, ICAM-1, LFA-1 and TNFα. Antagonist ligands are intended to include antisense nucleic acids, analogs or pharmacological antagonists, and antibodies.

EXAMPLE 2

Reduction of myoblast death by reducing the glycosylation of membrane glycoproteins.

The inhibition of intracellular oligosaccharide processing is a new approach to immunosuppression in allotransplantation. The net effect of such inhibition is reduction in the membrane expression of some glycoproteins. Hence cell-cell interaction in allorejection may be impaired in the presence of glycoprotein processing inhibitors because the expression of key ligand-receptor pairs of glycoproteins including

adhesion molecules is inhibited. Grochowicz et al. (1996, 1997) found that Castanospermine, an alpha-glucosidase 1 inhibitor, reduced the membrane expression of adhesion molecules (LFA-1 alpha, LFA-1, ICAM-1), class I and class II MHC antigens and on other T cell associated molecules (CD4, CD8, CD39, CD45, W3/13). All of these glycoproteins have N-linked carbohydrates. Grochowicz et al. (1996) and Hibberd et al. (1995 and 1997) also found that Castanospermine prolonged rat heart allograft survival in a dose-dependent manner and with limited testing was relatively non-toxic. Grochowicz et al. (1997) have shown that Castanospermine prolongs the survival of grafts in animals treated with FK506.

An alternative way to stop the interaction between the adhesion molecules and their receptors is therefore to block the formation of glycoproteins with Castanospermine. Castanospermine could also inhibit inflammation through its ability to prevent the expression of adhesion molecules, which may be necessary for the capture and retention of leukocytes in the inflamed tissue (Willenborg et al. 1992). Indeed, Castanospermine inhibits the passage of leukocytes through vascular subendothelial basement membranes by inhibiting the expression of leukocyte cell surface-bound enzymes that are essential for such migration.

The activation of neutrophils which is responsible for myoblast death requires the interaction between adhesion molecules such as LFA-1 and ICAM-1. The adhesions molecules are glycoproteins. It is possible that other unidentified glycoproteins are also involved in these cellular interactions. One way of blocking these interactions involving the cell surface glycoproteins is to block the glycosylation of proteins. This can be done with several drugs, one of them being Castanospermine.

<u>Effects of Castanospermine on myoblast oxidation *in vitro*.</u>

Experiment description:

Myoblasts were obtained from newborn CMV-LacZ transgenic mice, expressing a beta-galactosidase gene under a CMV promoter. These myoblasts were grown in culture during two days in presence of various concentrations of Castanospermine (1, 5 and 10 mM). The myoblasts were then stimulated with IL-1 during 6 hr. These cells were then loaded with a compound DCSF (Molecular Probes inc.) which becomes fluorescent following oxidation. Polymorphonuclear (PMN) cells were obtained from the abdominal cavity of mice injected with casein. They were activated with ZAS (Zymogen Activated Serum). These PMNs were added to myoblasts at a ratio of 5 PMNs per myoblasts. Fluorescence was read at every 15 min. during 60 min.

Results:

Figure 5 illustrates the results of the experiments. The control is made of myoblasts not treated with Castanospermine. The control fluorescence value is set at 100%. The reduced glycosylation produced by Castanospermine resulted in a marked reduction of the myoblast oxidation by the PMNs. This should therefore reduce the

death of the myblast *in vivo*.

Effects of Castanospermine on myoblast death *in vivo*:

Experiment description:

Four female CD1 mice received with an osmotic mini pump 0.855 mg/day of Castanospermine during 4 days, starting the day before myoblast transplantation. Myoblasts were grown from a muscle biopsy of CMV-LacZ mice. They were treated or not with 1 mM Castanospermine during 24 hours. Roughly 800,000 cells treated with Castanospermine were transplanted in the right Tibialis anterior muscle of 6 mice. In parallel 12 control mice not treated with Castanospermine were injected with 800,000 myoblasts not treated with Castanospermine. Six of these control mice were sacrificed after 1 hour. All the other mice were sacrificed after three days. Their right side was taken and frozen immediately in liquid nitrogen. The muscles were later homogenized and the beta-galactosidase activity was determined.

Results:

The enzyme activity at one hour was set at 100%. The percentage of beta-galactosidase activity at 3 days was calculated relative to the 1 hour result. Figure 6 illustrates the results. There was a 73% lost of enzyme activity in the control (not treated with Castanospermine) and a 64% lost of activity in those that received Castanospermine. The difference was significant. The reduced lost of beta-galactosidase activity was attributed to a reduction of myoblast death.

Effects of Castanospermine on myoblast fusion in culture.

Experiment description:

Dog myoblasts were grown in a fusion medium (MCDB with 15% FCS) with or without Castanospermine. The number of nuclei incorporated in myotubes was counted in each group.

Results:

Without Castanospermine there was an average of 2.6 +/- nuclei per microscopic field. With 1 mM Castanospermine there was 18 +/- nuclei per field. The inhibition of glycosylation produced by Castanospermine increased the myoblast fusion. This treatment will therefore improve the success of myoblast transplantation by not only reducing the myoblast oxidation but also by improving the fusion.

EXAMPLE 3

Effects of metalloproteases on the fusion of donor/host cells and the diffusion of donor cells into host tissue

Metalloproteases are enzymes involved in tumor invasion and migration of cells (Stetler-Stevenson et al FASEB J. 7: 1434-1441; 1993). There are also involved in extracellular matrix restructuration during tissue remodelling (Gaire et al, JBC, 269, no 3 pp.1032-2040 1994). Matrilysin and Gelatinase A are metalloproteases involved in tumor invasion (Sato et al, FEBS Letters, 385 (1996) 238-240). Gelatinase A have

been associated with muscular degenerescence and regeneration (Kherif et al, Colloque sur les maladies neuromusculaires). Gelatinase A induces cell migration by cleaving laminin-5, exposing a pro-migration kryptic site (Giannelli et al, Science 97 (277) pp.225-228). We have shown that concanavalin A, a lectin that stimulates the expression of metalloproteases, increases by 3-fold the migration of myoblasts of CMVLacZ mice into a recipient muscle time. TPA, a phorbol ester, also increases by 4-fold the muscle area comprising transplanted myoblasts. We demonstrated hereinbelow that the success of transplantation may be improved by injecting myoblasts which express matrilysin (MMP-7) as one example of a useful metalloproteases, which myoblasts are also conditionally immortalized by the expression of a thermosensitive mutant of SV40 large T antigen.

A three fold increase of intramuscular migration of myoblasts obtained from primary cultures was achieved by our group by the addition of concanavalin A to the culture medium. This augmentation was attributed to an additional MMP expression. MMPs are involved in the normal tissue turnover and remodulation during growth and development. To overcome extracellular matrix (ECM) barriers, advancing cells must secrete proteases such as MMPs or protease activators such as urokinase at their leading edge, where complex proteolysis can direct migration, preserve ECM attachment, and avoid unwanted tissue damage. It is still unclear whether proteases remove physical barriers or if they modify the ECM in such a way that it is suitable for migration. However, metalloproteinases are found in abundance in their inactive precursor form in normal tissues and fluids, suggesting that their activities are limited by physiological activation rather than by expression alone (37). These enzymes are made of common domains and fall into four main groups called the collagenases, gelatinases, stromelysins and membrane-type metalloproteinases. Their catalytic mechanism depends on a zinc ion bound at the active center; each enzyme is secreted with a propeptide which is removed proteolytically before the enzyme becomes active.

Matrilysin (MMP-7), originally described as a putative metalloproteinase (PUMP-1), degrades type IV collagen, laminin-1, fibronectin, proteoglycan, type I, III, IV, V gelatin and insoluble elastin. It is also known to activate collagenase (MMP-1) to its fully activated form. MMP-1 degrades type I, II, III, VII, X collagen and gelatins. MMP-7 can be purified in its inactive zymogen form of 28 kDa and as an active species of 19 kDa. It lacks the C-terminal domain common to all other metalloproteinases and is therefore the smallest.

The extracellular matrix has a profound influence on muscle morphogenesis. Although damaged muscle fibers regenerate from a resident population of satellite cells that are wedged between the basal lamina (BL) and the muscle fiber, these satellite cells apparently penetrate poorly the BL unless it is mechanically damaged or proteolytically digested. Laminin, fibronectin, proteoglycan, collagen I, III, IV, V, VI are

all associated with the muscle fiber BL. The BL limits the growth and migration of activated satellite cells, orients the regeneration of new muscle fibers and could be involved in repressing satellite cell mitosis and differentiation in the absence of damage. The aim of the present study was to investigate the effects of constitutive MMP-7 expression in mouse myoblasts on the migratory and fusogenic potential of these cells in the muscle tissue.

MATERIALS AND METHODS

Cells and cell cultures:

The parental Immortomyoblast cell line clone (clone #7) was derived from muscle biopsies of a newborn transgenic TnlLacZ immortomouse. The TnlLacZ immortomouse was obtained by interbreeding H-2k^b-tsA58 transgenic mice with TnlLacZ transgenic CD1 mice. H-2k^b-tsA58 transgenic mice harbor a thermolabile immortalizing gene (the tsA58 mutant of SV40 large T antigen) under the control of an inducible promoter (H-2k^b). The expression of SV40 large T antigen is induced through application of interferon gamma (INF-g). Immortomyoblast clones are grown in DMEM Low Glucose medium containing 15% Donor Calf Serum (Gibco), 2% Chick Embryo (Gibco/BRL, Burlington, Ontario, Canada), 20 U/mL interferon gamma (INF-g), 1% (10 000 U/mL) penicillin, 1% (10 000 U/mL) streptomycin in 8% CO_2 at 32°C in permissive conditions for the expression and function of the tsA58 gene product. Immortomyoblast clones were grown in DMEM High Glucose medium containing 15% Donor Calf Serum (Gibco), 1% (10 000 U/mL) penicillin, 1% (10 000 U/mL) streptomycin in 5% CO_2 at 37°C for non-permissive conditions. Human myoblasts expressing normal dystrophin were obtained from fresh biopsies of patients as previously described and grown in MCDB120. Human primary culture myoblasts from a 12-year-old female and cultures from a cloned human myoblasts isolated from a 13-month-old patient were used for co-transplantation studies.

Plasmids and cell transfection.

To express human matrilysin in an Immortomyoblast clone and to generate a control cell line, the matrilysin expression vector pbAprMUTM and the control vector pRSVneo were transfected using Lipofectamine (GIBCO/BRL). pbAprMUTM contains the neomycin selectable marker gene and a mutated form of the human matrilysin cDNA driven under the human beta-actin promoter. pbAprMUTM was provided by J.D. Knox and is derived from the eucaryotic expression vector pH-b-APr-neo-1. pRSVneo contains the neomycin selectable marker gene under the RSV promoter. One day before the transfection, 2 x 10^5 Immortomyoblasts (clone #7) were plated into 6 well plates and allowed to adhere overnight. Transfection of 2 mg of DNA with Lipofectamine was performed using standard procedures as specified (GIBCO/BRL). The medium was changed twice per week using complete medium containing 400 mg/mL of Geneticin (Gibco), beginning the selective treatment 72 hours after the

transfection. The transfection using the pbAprMUTM vector generated 13 Geneticin resistant colonies and the pRSVneo vector, 8 Geneticin resistant colonies. Respective transfectants were pooled and expanded for freezing, analysis and transplantation. Immortomouse myoblasts derived from the pbAprMUTM transfected cells were designated 7mat and pRSVneo transfected myoblasts 7neo, while the parental cell line was called clone #7.

In vitro behavior of the Immortomyoblast clones.

7mat Immortomyoblasts and controls were expended in permissive conditions permitting the expression of the T antigen, i.e., 20 U/mL interferon gamma (INF-g) and 32 °C. *in vitro* fusion in permissive conditions was confirmed by X-Gal staining of the cells and myotubes, since myotubes generated in these conditions are smaller and can be confused with myoblasts. To determine the fusion index of each Immortomyoblast cell line in non-permissive conditions, an equal number of each of the Immortomyoblasts cell lines were grown to 50% confluence in permissive conditions. They were submitted for 4 days to non-permissive conditions which did not permit the expression of the T antigen but which permitted fusion. Myotubes formed in these non-proliferating conditions were quantified by counting 5 microscopic fields from 4 duplicate wells.

Cell preparation and transplantation.

Six SCID and 20 Balb/C mice (all females between 5 and 8 weeks old) were used as hosts for these experiments. Both Tibialis anterior (TA) were used in Balb/C mice for the implantation of Immortomouse myoblasts pellets (clone #7, 7mat or 7neo), each containing $5x10^5$ myoblasts. SCID mice were used for the transplantation of mix pellets containing $1x10^6$ human primary culture myoblasts and $5x10^5$ Immortomyoblasts (7mat, 7neo or none). The day of transplantation, the myoblasts were detached using 0,125% trypsin and washed three times in HBSS. These cells were kept on ice until transplantation. Pellets were obtained by centrifugation at 6500 rpm for 5 minutes. The skin was opened to expose the TA muscle and the myoblast pellets, resuspended in 10 mL of Hank's balanced salt solution (HBSS), were slowly injected into the belly of the TA along the rostrocaudal axis. A 1/2 cc U-100 Insulin Syringe (Becton Dickinson, NJ) was used for these injections, applying low pressure while the muscle was examined for leakage. The skin was then closed with fine sutures. FK 506 (Fujisawa Pharmaceuticals Co. Ltd, Osaka, Japan) was administered daily at 2.5 mg/kg to immunosuppress Balb/C mice.

Muscle biopsy and preparation.

21 days after myoblast transplantation, mice were deeply anesthetized with 0.15 mL of a solution containing ketamine (10 mg/mL) and xylazine (10 mg/mL). They were then killed by intracardiac perfusion with 0.9% saline containing heparin (2 IU/mL). The TA muscles were dissected out and immersed in 30% sucrose solution

at 4°C for 12 to 18 h. The specimens were imbedded in OCT and frozen in liquid nitrogen. Serial cryostat cross sections (12 mm for immunochemistry and 25 mm for X-Gal staining) of the muscles were thawed on gelatin-coated slides.

Reverse transcriptase polymerase chain reaction (RT-PCR).

Total RNA was isolated from cultured cells using guanidine isothiocyanate (Trizol Reagent, Gibco BRL) according to the supplied protocol. A DNAse treatment was performed to avoid contamination by the cDNA from the vector in the PCR reaction. The reverse transcription was performed at 42°C for 1-hr using MMLV reverse transcriptase (BRL) on 500 ng of sample RNA as described previously. The cDNA was amplified in a Perkin Elmer Cetus Thermal cycler using Tth DNA polymerase (Boehringer Mannheim, Montreal, Canada). Multiplex amplification of the cDNA was performed, using the beta-Actin cDNA as an internal control for the amplification of the human matrilysin cDNA. Primer sequences for mouse beta-Actin cDNA were as follows : sense, 5'-GTGGCGCGCTCTAGGCACCAA-3'; antisense, 5'-CTCTTTGATGTCACGCACGATTTC-3' as previously described. Primer sequences for the human matrilysin were designed to amplify a 397 bp fragment from the cDNA according to the published sequence (GenBank/EMBL data bank accession number: L22519) and were as follow: sense, 5'-CAGATGTGGAGTGCCAGATG-3'; antisense, 5'-CCAAGTTCATGAGTTGCAGC-3'. The samples were amplified for 30 cycles with the following parameters: 94°C, 1 min.; 60°C, 1.5 min.; 72°C, 2 min. The primers amplified a 540 bp (b-Actin) and 397 bp (matrilysin) fragments.

Immunohistochemistry and β-Galactosidase staining.

Human dystrophin was detected using the NCLDys3 monoclonal antibody which does not react with mouse dystrophin. The sections were incubated after TBS/BSA rinses respectively: 1.5 h with NCLDys3 (dilution 1:10; Novocastra, New Castle Upon Tyne, UK), 1 h with a rabbit antimouse antibody biotin conjugate (dilution 1:250; Dako) and 1.5 h with streptavidin Cy3 (dilution 1:500; Sigma, St. Louis, Mo). β-Gal expression in muscle fibers (in serial cuts) was detected on slides containing 25 mm thick cross sections of TA muscles and myotubes (in cultured cells) following a 5 min. fixation in 0.25% glutaraldehyde and staining in 0.4 mM X-gal (Sigma) at room temperature in a dark box overnight (12-18h).

Image and statistical analysis.

Images were digitalized using a Pixera digital camera (Pixera Corp., Capertino, CA). The maximum area of circle occupied by β-Galactosidase positive fibers were measured using NIH Image analysis software. All area measurements and positive fiber quantifications were performed at 100X magnification. Serial cross sections containing the maximum amount of β-gal positive fibers in each muscle were used to quantify the number of positive fibers, since it most logically corresponds to the injection site. The surface (in mm^2) of the minimum circle containing all the β-gal

positive fibers was also determined on the same section. RT-PCR products densitometry was performed with NIH Image analysis software, using molecular weight marker VI (Boehringer Mannheim) as internal standard for curve fitting. Significant differences were evaluated using an analysis of variance (ANOVA) followed by a Fischer's PLSD test on a Stat View 512 software (Brain Power, Calabasas, CA) with a level of $p<0.05$ being considered significant.

RESULTS

Generation of matrilysin expressing Immortomyoblasts.

The transfection on the parental cell line, the Immortomyoblast clone #7, with pbAprMUTM vector generated 13 Geneticin resistant colonies (7mat). The transfection with pRSVneo yielded 8 Geneticin-resistant control clones (7neo). The resistant transfectants of each type were pooled together to avoid drifting from the parental cell line. Myoblast cultures obtained by the transfection of the matrilysin expression vector (7mat) and the control vector (7neo) were observed and compared with the parental cell line (clone #7). None of the stably transfected cell lines exhibited any significant modifications in their doubling time compared to clone #7 once the geneticin selection was stopped. While 7neo and clone #7 myoblasts maintained a similar usual morphology (small, round cells) in culture, 7mat myoblasts showed a vastly increased tendency to form myotubes spontaneously in complete culture medium even at low confluence. Such fusion between those Immortomyoblast clones is rarely observed and is mostly seen in highly confluent cultures or in differentiation medium.

Matrilysin expression by Immortomyoblasts.

7mat Immortomyoblasts and controls were tested for the expression of human MMP-7 by multiplex RT-PCR. Only 7mat Immortomyoblasts expressed the transgene as shown (Fig.1). Densitometry analysis showed a four fold difference in favor of the b-actin mRNA. Mouse matrilysin never interfered with our analysis, it was either not expressed by the parental cell line or our detection techniques were specific to the human mRNA.

in vitro behavior of the Immortomyoblast clones.

All the Immortomyoblast cell lines have been found capable of terminal *in vitro* differentiation. The respective fusion capacity of 7mat, 7neo and clone #7 Immortomyoblasts was first compared by growing cells in permissive conditions for the expression and function of the tsA58 gene product. While 7neo and clone #7 Immortomyoblasts rarely formed myotubes spontaneously in permissive and non-confluent conditions, 7mat Immortomyoblasts fused often, even at semi-confluency . Such a behavior was never observed with the parental clone or with any of the other Immortomyoblast clones previously isolated from the same transgenic TnILacZ immortomouse (unpublished data). To investigate their ability to attain final differentiation and fusion, 7mat Immortomyoblasts and controls were left in conditions

which did not permit the expression and function of the T antigen for 4 days before myotubes were counted. Myotubes were quantified by counting five microscopic fields from 4 duplicate wells. As observed previously, Immortomyoblasts cell lines (7neo), rendered resistant to G418 by their stable transfection using mammalian expression vector, displayed a decreased fusion index compared with their parental counterpart. Strikingly, 7mat Immortomyoblasts formed nearly three times more fibers per field then clone#7 ($p<0.0001$) and seven times more fibers then control cells ($p<0.0001$).

Transplantation of matrilysin expressing Immortomyoblasts and controls.

To evaluate the effects of matrilysin expression on graft success, the three Immortomyoblast cell types were transplanted at only one site in uninjured TA muscles (n=11) of adult mice. Twenty-one days after the transplantation of the Immortomyoblasts, serial sections of the injected muscles were stained with X-Gal. The sections containing the maximum amount of b-Gal-positive fibers were selected for statistical analysis. Positive fibers were counted in each serial section and the fusion efficacy of 7mat, 7neo and clone #7 Immortomyoblasts was then compared. The circular area including all b-Gal-positive fibers of all TA muscles was also studied. 7mat Immortomyoblasts formed significantly more ($p<0.05$) b-Gal-positive fibers. However, the surface over which 7mat Immortomyoblasts formed fibers was large, but did not reach the significance level ($p=0.11$) compared with clone #7 Immortomyoblasts. Unlike clone #7 and 7mat, the control 7neo Immortomyoblasts did not generate any b-Gal-positive fiber in any of the 11 injected TA muscles (Fig. 3). The difference between 7mat and 7neo Immortomyoblasts was consequently highly significant ($p<0.001$) for the number of b-Gal-positive fibers generated and the covered surface (Figure 7). Figure 7 also shows that transgenic mouse myoblasts transfected with TnIβ-gal had a significant increase in the β-gal positive muscle fibers after activation with phorbol ester. The same results are observed with concanavalin A (not shown). These agents stimulate the production of metalloproteases. The diffusion of transplanted cells is better (3 to 4 fold increase) with concanavalin A that with MMP-7 probably because other metalloproteases are produced in response to concanavalin A.

Co-transplantation of matrilysin expressing Immortomyoblasts with primary human myoblast cultures in SCID mice.

In an attempt to investigate whether the effect on myoblast fusion was attributable to the proteolytic activity of secreted matrilysin but not to an intracellular modification only influencing the selected Immortomyoblast cells, matrilysin expressing myoblasts were co-injected with human myoblasts. This has also given us the opportunity to evaluate the potential of matrilysin to increase graft success for cells of a human origin. A human myoblast primary culture from a twelve year old female was co-transplanted in SCID mice TA muscles (n=4) with and without Immortomyoblasts expressing matrilysin (7mat). Serial sections revealed a highly increased amount of

fibers and myotubes expressing human dystrophin observed in the presence of cells expressing matrilysin, demonstrating the potential of matrilysin to influence the behavior of human myoblasts in a muscle. A six fold increase ($p<0.001$) in the number of fibers or myotubes expressing human dystrophin and a four fold increase ($p<0.05$) in the regenerated surface was obtained. Positive normal size fibers and very small fibers or myotubes were counted separately as shown. While cells expressing the matrilysin transgene increased by a factor six ($p<0.001$) the amount of human dystrophin positive myotubes and smaller fibers, the amount of human dystrophin positive fibers only doubled, not reaching a significant level ($p=0.3$). Most fibers generated contained both β-Gal activity (due to the fusion of 7mat) and human dystrophin (due to the fusion of human myoblasts). However, the β-Gal activity seemed less intense in co-transplanted muscles than in muscles only injected with Immortomyoblasts. This suggests that Immortomyoblasts have a limited contribution to the regenerative process compared with the human myoblasts.

DISCUSSION

The purpose of our study was to evaluate whether matrix degrading enzymes could stimulate the migration and the fusion of transplanted cells, therefore increasing the number of fibers expressing the donor phenotype. Our results demonstrated that the expression of a metalloproteinase targeting the extracellular matrix found in the muscle tissue promotes the formation of fibers expressing the donor genes on a greater surface. The extent by which we can increase the fusion capacity of myoblasts by overexpressing metalloproteinases is still unclear, since myoblasts seem to loose the capacity to form myotubes and fibers in the muscle following geneticin selection. The important loss in the ability of the 7neo Immortomyoblast cell line to form myofibers *in vivo* is not an isolated event, previous attempts to genetically engineer Immortomyoblasts(data not shown) also produced cell lines with a lowered capacity to fuse with host fibers following transplantation. In contrast with previous experiments, myoblasts genetically engineered to express MMP-7 gave promising results. However, avoiding G418 selection might give better results in future experiments of this type.

The exact mechanism by which matrilysin is able to promote graft success in this system is not yet determined. The increase in graft success by matrilysin expressing cells could be explained by numerous potential mechanisms divided in three groups. First, the localized degradation of the ECM surrounding the injected cells could stimulate myogenesis. Myoblasts are normally anchored to the ECM through integrins. Therefore the degradation of the ECM could increase cell-cell or cell-fiber interactions. Myoblasts could penetrate more easily through the basal lamina of the host muscle fibers and generate more hybrid myotubes following the partial degradation of the ECM. Recently meltrin-alpha, a metalloprotease-desintegrin found on myoblast surface, was shown to confer increased fusogenic properties and to be

essential for myotube formation. Thus, it is likely that muscle cell fusion involves metalloproteinase-sensitive mechanisms, as previously suggested.

Secondly, an increased amount of transduced fibers could result from the increased migration of transplanted myoblasts following the degradation of the ECM. The matrilysin activity might diminish the physical impairment of the ECM, therefore augmenting the diffusion area covered by the grafted cells. The modification of the ECM in a structure promoting the migration of the myoblasts over their quiescent embedding into the matrix could also explain increased cell diffusion. Several experiments have shown that the nature of the ECM on which a myoblast is attached can affect its motility. It has been shown that laminin-1 specifically stimulates myoblast proliferation, locomotion and myogenesis in skeletal muscle. Also, increased dispersion of myoblasts could be achieved by exposing promigratory peptides hidden in ECM proteins based in the BL and other ECM components in the muscle tissue. Recent results indicate that an epitope on the alpha-3 subunit of laminin-5, not being involved in adhesion, directly stimulates cell motility once it is functionally unmasked by MMP-2 cleavage.

In addition, new data suggest that the matrilysin activity is not restricted to the extracellular matrix. There are indeed evidences suggesting that MMPs are involved in the processing of TNF-alpha, heparin-binding epidermal growth factor, and Fas ligand. Therefore it has been speculated that matrilysin may activate lumenal or membrane-bound cytokines or growth factors, such as HGF or FGFs, to perturb locally the growth of the responding cells. Because of the complex interactions between the grafted myoblasts and the living tissue, we cannot clearly evaluate the relative importance of the potential mechanisms listed above.

However, the knowledge of the substrates for metalloproteases allows the prediction that the substrates themselves may be used *in lieu* of metalloproteases. The substrates may be used directly in the injectable compositions, or cells entering the compositions may be genetically engineered to produce the substrates (and metalloproteases, if necessary), during their *in vitro* culturing and/or their *in vivo* transplantation.

Our *in vivo* and *in vitro* results suggest new hypotheses on the apparently low percentage of injected myoblasts participating into muscle regeneration. Other teams have suggested that only a small amount of myoblasts are actually stem cells capable of surviving and proliferating in the muscle. Also, the myogenic potential of cultured myoblasts declines through multiple passages, indicating that cells with less myogenic potential are being selected. The important increase in the number of fibers expressing donor genes in the presence of matrilysin might indicate that this decrease in regenerative potential is not irreversible. Non cultured, freshly isolated myoblasts were shown to form more fibers, divide faster and migrate more then those cultured *in vitro*

before their transplantation. However, genetically modified autotransplantation or MHC compatible donor derived myoblast transplantation would still require *in vitro* culture to generate enough number of cells to achieve therapy. Also, while efficient myoblast transfer has been achieved in mechanically or chemically injured mouse muscles, the incorporation of myoblasts into unprepared muscles is still less efficient. Since the basal lamina and the surrounding connective tissue are more developed in dystrophic muscles then in their normal counterparts, it may constitute a barrier to high efficiency incorporation of exogenous myoblasts. This phenomenon could be of greater consequence in Duchenne patients, where connective tissue formed by and with fibroblasts occupies a greater surface. The expression of matrilysin in the surroundings of grafted cells could prove to be of significant help, not mainly in helping the migration of the myoblasts but in increasing the regenerating myogenic capacity of myoblasts after multiple passages. The use of metalloproteinase transgenes or ECM degrading enzymes and/or their substrates could possibly reduce the efficiency gap observed between freshly isolated and multiple passage myoblasts.

It is worthwhile noting that many other members of metalloprotease family exist. This invention is therefore not limited to the use of MMP-7 as a putative useful enzyme. The compositions of this invention therefore include one or more of gelatinase A (MMP-2), gelatinase B (MMP-9), collagenase-1 (MMP-1), collagenase-2 (MMP-8), collagenase-3 (MMP-13), collagenase-4 (MMP-18), stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), stromelysin-3 (MMP-11), metalloelastase (MMP-12), matrilysin (MMP-7), MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT2-MMP (MMP-16), MT3-MMP (MMP-16) and MT4-MMP (MMP-17). They may also include inducers like concanavalin A, phorbol esters and urokinase.

EXAMPLE 4

Effects of a myotoxic agent and of multiple sites injection on the success of myoblast transplantation in monkeys and the effect of replacing the administration of a myotoxic agent with a pretreatment of donor myoblasts with a growth factor.

Animals

The donor myoblasts may be grown from a muscle biopsy of an histocompatible or an histo-incompatible mouse. In the cases where myoblasts are obtained from an histoincompatible donors, the host may be immuno-suppressed with cyclosporin, but more effective immuno-suppression may be obtained with rapamycin, FK506 (Kinoshita et al. 1994; Vilquin et al. 1994, 1995) or monoclonal antibodies against molecules located on the membrane of lymphocytes or of antigen presenting cell, as described above.

Myogenic cells have been shown to be able to form new or hybrid (containing donor and host nuclei) muscle fibers in monkeys. The injected myoblasts can be

obtained either from the animal itself or from another animal. In both cases, the host has to be adequately immunosuppressed. Immunosuppression is required at least for a short term because foreign antigens are present in the culture medium. This experiment demonstrated that new muscle fibers can be formed in a primate. In this experiment, the injected cells were labelled with a retrovirus vector containing the β-galactosidase gene. The muscle fibers formed partially or totally by the injected myoblasts expressed that gene demonstrating that new genes can be effectively introduced in muscle cells of primates by myoblast transplantation.

In this experience four monkeys (*Macaca mulata*) were included as recipients for allotransplantations of myoblasts (#61, #62, #63 and #64).

Myoblasts originate from primary myoblast cultures obtained from muscle biopsies taken from two monkeys approximately two weeks before the transplantation. Biopsies were performed in the quadriceps femori by opening the skin and aponeurosis and exposing the belly of the muscle under general anesthesia. A biopsy of approximately 0.5 cm^3 was taken and thereafter the muscle, the aponeurosis and the skin were closed separately with absorbable sutures.

The biopsies were dissociated as in the routine, cells were cultured in MCDB supplemented with 15% of bovine calf serum and infected three times with the retrovirus LNPOZ, containing a β-galactosidase reporter gene. The final percentage of cells expressing the β-galactosidase was approximately 30%.

All the transplantations were allogeneic. The number of cells transplanted by site were $4x10^6$ (one site) $8x10^6$ (two sites) and 24x10 (ône site). For the transplantation the final pellets of cells were resuspended in 100 µl of HBSS ($4x10^6$, $8x10^6$ and $24x10^6$ cells) or in 100 µl of notexin diluted 5 µg/mL in HBSS ($8x10^6$ cells).

For each animal four sites of transplantation were performed simultaneously and the muscles used for transplantation were both biceps brachii and both quadriceps femori. Three muscles received respectively $4x10^6$, $8x10^6$ and $24x10^6$ cells in HBSS and one muscle $8x10^6$ cells resuspended in notexin. The transplantation was performed by opening the skin and aponeurosis and exposing the belly of the muscle. The injection of cells was performed using a Hamilton syringe. Cells were injected obliquely or almost longitudinally into a region of approximately 5-8 mm just under the surface of the muscle and covering an area lesser of 1 cm^2. 3 µl of cell suspension was carefully injected during the needle withdrawal at each injection point and roughly 35 to 50 injections were performed by no more than 10 sites of puncture (several injections were performed by the same puncture). Care was taken to perform each injection very close to the other. The site of transplantation was identified by placing two points of non-absorbable suture comprising the region of the transplantation, one proximal and one distal to it to identified it at the biopsy. The aponeurosis and skin were closed separately with absorbable sutures.

The monkeys were immunosuppressed intramuscularly with FK506. FK506 was given at 0.5 mg/kg from three days before the transplantation to three days after and therefore the dose was diminished to 0.15 mg/kg. Nevertheless, in two monkeys the FK506 was increased to 0.3 mg/kg 10 days after the transplantation, taking into account that cell infiltration was seen in the other two monkeys when the first biopsies were analyzed. Four weeks after the transplantation, the sites grafted were biopsied under general anesthesia in the same way as it was described for the culture of cells.

The biopsies of muscles were placed in sucrose 30% overnight at 4°C and thereafter frozen in liquid nitrogen. Sections of 30 μm and 10 μm were performed in a cryostat at -25°C.

Serial sections were stained to show different histological and histochemical features. Hematoxylin- eosin stain was performed to show the general histology of the grafted muscle (eventual presence of cell infiltration and central nucleus as evidence of regeneration). β-Gal in transplanted cells was revealed. Hematoxylin-eosin slides were mounted in Permount and X-Gal slides mounted in a glycerin-gelatin medium.

Results:

The number of β-gal positive muscular fibers counted in the best section are represented for each monkey:

Monkey	$4x10^6$	$8x10^6$	$24x10^6$	$8x10^6$ + notexin
61	636	485	1354	1444
62	996	1434	1285	3782
63	863	1570	905	3938
64	795	1523	791	2244

It must be signaled that the extension of the largest β-gal staining in the sections was found in almost 3 to 4 mm of the fiber extension, overall when notexin was used, and that β-gal expression, although variable, is remarkable important in the largest proportion of the fibers.

These results clearly indicated that better transplantation results are obtained in monkeys when there is a pretreatment with notexin. Since treatment of patients with a toxin may not be clinically acceptable, and since I previously demonstrated that growing myoblasts in the presence of a trophic factor such as βFGF is a valuable alternative to a toxin treatment, it is reasonably inferred that trophic factors would advantageously replace notexin. Moreover, the injection at multiple sites may be replaced in part by the use of metalloproteases and/or inhibitors of glycoprotein formation which encourage the diffusion of transplanted cells into the host tissue.

EXAMPLE 5

Transplantation SV40 of large T antigen - immortalized myogenic cells

The C57BL/6J *dy/dy* mice (Jackson Lab.) were used as recipients for myoblast transplantation. The C57BL/6J +/+ normal newborn mice (Jackson) were used as compatible donors for some myoblast transplantations. These strains are used to study the effect of the marker LacZ gene. The marker gene is an indicator of transformation success. In clinical trials, the protein may be, factor VIII, merosin, dystrophin, connexin, etc. depending on the disease to cure.

The transgenic TnI-LacZV29 mice (Tn-LacZ, gift from K. Hasting, McGill University, Montreal, Canada) contain the LacZ gene under the control of the quail fast troponin I promoter, thus, differentiated muscle cells express a cytoplasmic β-galactosidase β(-gal) protein (Hallauer et al., 1993; Kinoshita et al., 1994a,b). β-gal expression is not restricted to the nucleus in this model. Both male and female parents are heterozygous animals, and newborn mice were used as donors for some immunologically noncompatible myoblast transplantations.

The H-2k^b-tsA58 transgenic mice carry the thermolabile tsA58 mutant of SV40 large T antigen under the control of the H-2K^b promoter (Jat et al., 1991). Interferon γ (IFN-γ) increases the transcription of this promoter. The thermolabile protein is functionally active at 33°C but not at 39°C. These characteristics facilitate the derivation of conditionally immortalized cell lines (Morgan et al., 1994). An homozygous H-2Kb-tsA58 male mouse (Charles River Lab., Wilmington, MA) was crossed with a heterozygous transgenic Tn-LacZ female mouse. Offsprings were all heterozygous for the H-2Kb-tsA58 transgene and some were heterozygous for the Tn-LacZ transgene. Thus, newborns were tested for β-gal expression, and only β-gal positive animals were used for the establishment of myogenic cell lines (see below).

Primary cell cultures

Mouse primary myoblast cultures were obtained from biopsies from newborn skeletal muscle as previously described (Vilquin et al., 1995c). The cell suspension was cultured in 199 medium (Gibco, Grand Island, NY) supplemented with 15% FBS (Gibco) and antibiotics. Cells were harvested at 70% confluence, that is, 2 days after plating, either for immediate grafting or for freezing until grafting. These cultures were not pure and contained several cell types, with 30% to 40% being committed myoblasts as assessed by desmin immunostaining (personal results).

Cultures were also obtained from newborn transgenic Tn-LacZ. Because parents were both heterozygous, newborns were individually tested for the expression of β-gal using X-gal (see below). Only β-gal-positive newborns were used for primary myoblast cultures.

Different batches of primary cell cultures have been used for this work. The mice transplanted with these batches have been gathered under the letters A, B (β-gal cells), and H (histocompatible cells).

Establishment of permanent myogenic cell lines from (TnI-LacZ1/29J X (H-2Kb-tsA68)

offsprings.

Primary muscle cell cultures were started using the β-gal-positive offsprings. Following preplating, the cells were plated at 50 cells/cm^2 in gelatin-coated wells and grown in DMEM (Gibco) supplemented with 20% FCS and 2% chick embryo extract (Gibco) at 33°C in 10% CO_2. Mouse recombinant IFN-γ (Genzyme Co., Cambridge, MA) was added at the final concentration of 20 U/ml (Morgan et al., 1994). Colonies of typical myogenic morphology were subsequently cloned in 96-well plates at the limit dilution of 1 cell per well. The myogenicity of colonies and clones was assessed by desmin immunostaining and by the ability to fuse and form myotubes *in vitro*. Fusion was obtained by reducing the FCS concentration to 5% and growing the cells at 37°C in 5% CO_2 in the absence of IFN-γ.

The problem of tumorigenicity was frequently reported upon the use of the classical C2 mouse myoblast cell line (Wernig et al., 1991; Morgan et al., 1992). Thus, Morgan et al. (1994) developed a technique to obtain pure, immortalized myoblast clones with the ability to form new muscle fibers after transplantation into immunodeficient and dystrophin-deficient *nu/mdx* mice, without inducing tumors. The transgenic myoblast cultures developed in the present study have the additional advantage that differentiated cells express β-gal under the control of a specific muscle promoter. This allows the rapid identification of new or hybrid fibers formed by the fusion of these myoblasts *in vitro* or *in vivo* in any mouse model.

The muscle cells isolated as colonies or single clones from newborn muscles of (Tn-LacZ) X (H-2K^b-tsA58) mice were able to grow and proliferate at 33°C in a 10% CO_2 atmosphere when stimulated by murine IFN-γ. When shifted to differentiation medium the muscle cells fused together and formed giant myotubes. These cells, either myotubes or myoblasts alone, expressed the intracellular filament desmin, which is an early marker of myoblasts (Lin et al., 1994). These myotubes and some myoblasts expressed also β-gal. These cells were able to fuse and to form new or hybrid muscle fibers expressing β-gal *in vivo* in immunodeficient SCID mice without forming solid tumors. The colony # 24 (two cells in the original cloning well) and the clones MB3, MB7 and MB27 were selected based on their *in vitro* myogenic characteristics and were injected into *dy/dy* muscles.

Cell transplantation

On the day of transplantation, the cells were harvested by trypsinization or thawing, washed three times in HBSS (Gibco), and concentrated as pellets. Cell viability was assessed using trypan blue staining. The Tibialis anterior (TA) muscles were exposed and injected with approximately 4 x 10^6 (primary muscle cell cultures), or 10^6 (myogenic cell lines # 24, MB7, MB27) or 3 x 10^6 (myogenic cell line MB3) viable cells suspended in 10 μl of HBSS. When the role of myoblasts in LAMA2 restoration was explored by clonal cell transplantation, the left TA of the mouse received only

myogenic clones, while the right TA received both myogenic clones and a histocompatible primary muscle cell culture (i.e. obtained from normal C57BL6J+/+ newborn mice). Some protocols included γ-irradiation and/or notexin treatment of the muscles before cell transplantation: three days before transplantation, one or both hind legs of the *dy/dy* mice were Cobalt-irradiated (20 Gy). This level of irradiation has been shown to block host myoblast proliferation and to favour donor myoblast implantation (Wirtz et al., 1982; Morgan et al., 1990; Wakeford et al., 1991); one day before transplantation, one or both TA were exposed and injected with 10 μl of notexin venom (5 μg/ml), which has been shown to trigger muscle fiber degeneration without damaging myoblasts (Harris et al., 1975). FK506 immunosuppression was started on the day of transplantation in noncompatible grafting models (2.5 mg/kg/d i.m., Fujisawa Co, Osaka, Japan; Kinoshita et al., 1994b).

Muscle collection

At the times indicated, the TA muscles were collected and immersed in a sucrose solution (Tremblay et al., 1993). Muscles were embedded, frozen in liquid nitrogen, and serially sectioned at 8 μm. Adjacent serial sections were thus spaced by 8 μm, while the series of sections were separated by 180 μm.

β-*gal histochemistry*

Differentiated conditionally immortalized myogenic cells and muscle cryostat sections were fixed with 0.25% glutaraldehyde for 3 min and incubated overnight at room temperature with 0.4 mM X-gal (5-bromo-4-chloro- 3-indolyl--D-galactopyranoside, Boehringer Mannheim, Laval, Canada) (Kinoshita et al., 1994a). Preliminary experiments indicated that the X-gal enzymatic histochemistry was as sensitive as three-step immunohistochemistry using monoclonal anti--galactosidase antibodies to localize muscle fibers formed by the fusion of donor myoblasts (not shown). Newborns originating from Tn-LacZ mice were tested for β-gal expression using the X-gal reagent, except that no fixation was necessary and incubation of a small muscle piece was performed at 37°C for 1 h.

LAMA2 immunohistochemistry

Muscle sections were fixed in acetone at -20°C for 10 min. then nonspecific Ig binding was blocked with 10% FBS in PBS for 30 min. The rabbit polyclonal anti-mouse LAMA2 (Xu et al., 1994a) was used 1/300 in PBS containing 1% FBS for 2 h at 37°C. The second antibody was a biotinylated goat anti-rabbit Ig (1/100 in PBS containing 1% FBS for 1 h; Dako, Copenhagen, Denmark). The next step was incubation with streptavidin-HRP or streptavidin- FITC in some cases (1/200 in PBS containing 1% FBS; Dako). Binding was revealed with DAB (0.5 mg/ml, Sigma) and 0.015% hydrogen peroxide. Slides were mounted in PBS-glycerol. Immunoperoxidase-positive and -negative fibers were counted by microscopic examination of each muscle on the section with the most positive fibers.

Desmin immunocytochemistry

Conditionally immortalized myogenic cells were grown on 2% gelatin-coated plates and allowed to fuse in 5% FBS at 37°C. The cells were fixed and permeabilized with methanol at -20°C . Nonspecific binding was blocked using 10% FBS in PBS for 30 min. The cells were incubated with a mouse anti-desmin antibody (1/50 in PBS containing 1% FBS for 1 h; Dako). The second antibody was FITC-conjugated rabbit anti-mouse IgG (1/100 in PBS containing 1% FBS for 1 h; Dako).

Primary muscle cell culture transplantation under FK506 immunosuppression

<u>β-gal expression</u>

Primary muscle cell cultures from transgenic mice expressing β-gal under the control of a muscle-specific promoter were able to develop inside the TA muscles of *dy/dy* mice immunosuppressed with FK506. Some myoblasts fused together or with host muscle fibers to form new or hybrid muscle fibers expressing β-gal. These β-gal expressing fibers were not numerous and they were not dispersed throughout muscles. They were presumably located only near the injection sites. As seen in Table I, the percentage of β-gal-positive fibers was rarely higher than two. These low percentages were obtained whatever the age of the recipient mice at the time of transplantation, a slight but not significant increase was observed when animals were kept longer after transplantation.

<u>LAMA2 expression</u>

As previously reported, LAMA2 was absent from muscles of *dy/dy* mice (Arahata et al., 1993; Sunada et al., 1994; Xu et al., 1994a, b) and was never expressed in muscles only injected with HBSS. LAMA2 surrounded normal mouse muscles. LAMA2 was expressed around some muscle fibers following Tn-LacZ primary muscle cell culture transplantation. The total and relative numbers of LAMA2-positive fibers, however, depended on the age of the mouse at transplantation and on the pretreatment of the muscle . The percentage of LAMA2-positive fibers was low (mean ± SD, 6.4 ± 4.4) when older animals (i.e., more than 3 months), were used as recipients. The percentage of LAMA2-positive fibers was higher (15.9 ± 9.0) when younger animals (i.e., 6 week old) were used as recipients. Notexin alone, or γ-irradiation alone, did not increase the number of LAMA2-positive fibers. The combination use of notexin and γ-irradiation increased the percentage of LAMA2-positive fibers in transplanted muscles (27.8 ± 14.9). There was an overall but not significant increase in the percentage of LAMA2-positive fibers with the duration of the experiment (the mice in group A all received the same myoblast preparation).

<u>Colocalization of β-gal and LAMA2</u>

The Tn-LacZ primary muscle cultures originate from mice with normal LAMA2 expression. Thus, both the normal gene for LAMA2 and the reporter gene coding for β-gal are present in the donor cells. LAMA2 and β-gal were characterized on serial

sections only spaced by 8 μm. The percentages of LAMA2-positive and β-gal positive fibers were comparable when old animals (more than 3 months) were used for transplantation. LAMA2 and β-gal labeling were localised in the same clusters of fibers. Some LAMA2-positive fibers, however, were negative for β-gal expression, and some β-gal positive fibers were negative for LAMA2 expression on serial sections. Most of the smallest fibers were β-gal positive. These results indicated that β-gal and LAMA2 expression were not regulated similarly, or that the same type of cells were not responsible for their expression.

When experiments were designed in younger animals (i.e. 6 weeks old), the LAMA2-positive fibers were much more abundant than β-gal positive fibers. Surprisingly, the pattern and intensity of β-gal and LAMA2 expression greatly differed. Some LAMA2-positive clusters of fibers were totally devoid of β-gal. The β-gal positive fibers were restricted to smaller areas than LAMA2-surrounded fibers. LAMA2-positive fibers were observed over long distances throughout the muscle, i.e. more than 1000μm, whereas no β-gal was observed in most of these fibers over all length of LAMA2 expression.

<u>Long-term histocompatible transplantations</u>

Transplantation of histocompatible, isogenic primary mouse muscle cells in 6 week-old male *dy/dy* mice led to long-term LAMA2 expression (i.e. 11 weeks) in the transplanted muscles of all the animals. The percentage of LAMA2-positive muscles was greater when muscles were treated using notexin and γ-irradiation before transplantation (14.75 ± 6.4 without pretreatment versus 41.2 ± 14. 3 with pretreatment). Notexin alone (12.75 ± 1.2 positive fibers), or irradiation alone (19.25 ± 6.9 positive fibers), followed by transplantation, was not sufficient to increase dramatically the percentage of LAMA2-positive fibers. Given the small number of *dy/dy* mice available for these experiments, however, no statistical analysis was performed.

LAMA2 seemed to have spread centrifugally from the center of the injection site to its periphery. While LAMA2 completely surrounded normal mouse muscle fibers, its localization was frequently incomplete and disrupted in transplanted *dy/dy* muscles, especially in case of the largest fibers. Thus, LAMA2 deposition seemed to be localized. LAMA2 deposition also presented important variations in transplanted mice as compared to normal mice. Most of the smallest fibers were totally surrounded by LAMA2.

Overall transplantation success

Three transplantation models were used in this study. In the first one, nonhistocompatible primary muscle cells expressing the β-gal under control of a muscular promoter were transplanted into *dy/dy* mice. This allogenic transplantation required an efficient immunosuppression, which was obtained using FK506 (Kinoshita et al., 1994b; Vilquin et al., 1995b). The use of β-gal expressing cells showed the

exogenous origin of the labelled muscle fibers and allowed the comparison between LAMA2 and β-gal localization after transplantation. In the second model, histocompatible syngeneic primary cultures from normal littermates were transplanted into *dy/dy* mice. This model did not require immunosuppression and allowed the study of muscle regeneration over longer time. In the third model, pure but nonhistocompatible myoblasts were transplanted into one leg of FK506-immunosuppressed *dy/dy* mice, whereas the other leg received histocompatible primary culture as a control of LAMA2 expression. This model allowed the direct comparison of the outcome of transplantation between pure myoblasts and primary muscle cells and allowed the investigation of the potential role of myoblasts in LAMA2 deposition. Cell transplantation allowed the restoration of a structural, extracellular protein. In these immunologically controlled models the transplantation of primary muscle cells lead to LAMA2 expression in variable amounts in all animals. The number of LAMA2-surrounded fibers was shown to depend on the age of the animal at the time of transplantation and on the muscle pretreatment. The highest numbers of LAMA2-surrounded fibers were obtained when young animals (6 week old) received histocompatible cells after γ-irradiation and notexin pretreatment of the muscle. Irradiation was shown to hamper host myoblast proliferation and thus reduce normal muscle regeneration, and to favour extensive fibrosis in *dy/dy* (Wirtz et al., 1982) and *mdx/mdx* (Wakeford et al., 1991) mice. This treatment greatly increased donor myoblast permeation into host degenerating fibers and the formation of new or hybrid dystrophin-positive fibers in *mdx/mdx* mice (Morgan et al., 1990). In our models, it is thus likely that irradiation and notexin necrosis favour donor cell development *in vivo* over the *dy/dy* recipient cells.

In the histocompatible model LAMA2, which is the only known difference between *dy/dy* host mice and +/? donor mice, is not sufficient to trigger efficient acute rejection of LAMA2- expressing cells. Chronic rejection was not assessed in this study, but the overall highest numbers and percentages of LAMA2-surrounded fibers were observed 11 weeks after grafting. LAMA2 is undetectable in immunohistochemistry but very low amounts of LAMA2 mRNA are detected in untreated *dy/dy* mice by RT-PCR (Arahata et al., 1993; Xu et al., 1994a). These small amounts of LAMA2 would be sufficient to make the animals tolerant to their self antigens and to avoid immunological rejection of the transplanted LAMA2.

The intensity of β-gal staining and the number of β-gal positive fibers after transgenic Tn-LacZ culture transplantation was low as compared to results obtained in other, non *dy/dy* mice strains under the same FK506 immunosuppressive treatment. In a previous report, up to 90% of *mdx/mdx* TA muscle fibers could express β-gal only one month after transplantation (Kinoshita et al., 1994b). The present results suggest that muscle cell transplantation is less efficient in *dy/dy* mouse than in *mdx*. Actually,

more than 1000 dystrophin-positive fibers are frequently obtained in only 2 months following histocompatible primary muscle culture transplantation in *mdx/mdx* mice (Vilquin et al., 1995c), whereas it is difficult to obtain more than 300 to 400 laminin-2-surrounded muscle fibers in the same time in *dy/dy* mice. The *dy/dy* muscle fibers are smaller than normal muscle fibers and they show important size variation within a single muscle. This may reflect trophic problems in *dy/dy* muscles related to the absence of LAMA2 in basal lamina that could explain the variation in efficiency between the *mdx/mdx* and the *dy/dy* models. First, the initial lack of LAMA2 could hamper myoblast migration or alignment either with other myoblasts or with host muscle fibers, because basal lamina is known to play important roles in the control of migration, proliferation and differentiation of various cell types (Engvall et al., 1992). Second, an impaired muscle innervation due to the absence of LAMA2 around peripheral nerve fibers or neuromuscular junctions (Leivo and Engvall, 1988; Sunada et al., 1994; Xu et al., 1994a) could induce a muscle atrophy. Third, the myoblast proliferation and/or migration could be impaired by the abundant connective tissues, present especially in the oldest *dy/dy* mice. Taken together, these problems may explain why muscle regeneration was relatively poor following transplantation, whatever the pretreatment used.

LAMA2 and β-gal colocalization after transplantation of transgenic primary cells

In transplanted muscle, LAMA2 was generally more widely distributed than β-gal. This could indicate differences in the regulation of expression of these two proteins, that LAMA2 but not β-gal is diffusible, or that the cell type responsible for LAMA2 expression is not the cell type responsible for β-gal expression. β-gal expression is restricted to skeletal muscle cells as an intracellular protein, and its nuclear domain is about 1000 μm. In contrast, laminins and likely LAMA2 are secreted outside of the cells, and may have a relatively long half-life *in vivo* (Engvall, 1993) as components of muscle cells basal lamina. Thus, the fate of β-gal and LAMA2 *in vivo* is probably different. Whereas β-gal expression requires myoblast fusion and formation of hybrid or new muscle fibers, the developmental mechanisms of LAMA2 expression are still unclear.

LAMA2 expression pattern

LAMA2 generally deposited continuously around small caliber muscle fibers, whereas this deposition was frequently discontinuous around normal diameter muscle fibers, even two months after transplantation. In contrast, dystrophin was expressed along the complete inner circumference of muscle fibers by some weeks after transplantation, and dystrophin disruption was rarely observed one or several months after transplantation in the *mdx/mdx* or SCID mouse models (Partridge et al., 1989; Morgan et al., 1990, 1993, 1994; Huard et al., 1994; Kinoshita et al., 1994b; Vilquin et al., 1995c). The expression of LAMA2 seemed to be centrifuge relative to injection

sites. This observation suggests that competent cells could proliferate and diffuse, or that the secretion product may diffuse, thus producing a gradient of LAMA2 expression from center to periphery, that would progressively accumulate around the most proximal muscle fibers expressing the appropriate receptors and extracellular matrix components, allowing LAMA2 sequestration around these muscle fibers. This hypothesis could explain the polarization of LAMA2 deposition around the biggest fibers in the particular orientation from periphery to center. Small-diameter fibers would be more completely surrounded by LAMA2 because they were located at the injection sites in close contact with LAMA2-secreting cells. The surface to be covered by LAMA2 is also less in small-diameter than in normal-diameter fibers.

The transplantation models and results presented above should prove useful to study *in vivo* interactions between muscle cells and extracellular matrix, and interactions or coordinate regulations between nerve and muscle cells. These models should allow to study the regulations of extracellular matrix protein interactions *in vivo*. The critical steps for normal and pathologic muscle regeneration, that is, myoblast proliferation, alignment or fusion, or the stability of nerve-muscle regulations, should be discriminated.

The above results suggest that the *dy/dy* mouse develops a pathology of the extracellular matrix. Some clinical signs of this matrix pathology are similar to those observed in some neuromuscular diseases primarily originating from muscle or nerve cell deficiencies. These observations should prove useful in the investigation of human CMD aetiology.

It may be possible to develop new strategies for the correction of muscular diseases by gene complementation. The technologies will differ from those used for myoblast culture and transplantation in other types of muscular dystrophies if LAMA2 or any other extracellular matrix protein, may be secreted by another cell type which could be for example fibroblasts, macrophages or neutrophils genetically modified to secrete an extracellular matrix protein. Long-term experiments should also be designed to further explore the feasibility of myelination restoration in the peripheral nervous system of the *dy/dy* mouse by these genetically modified cells (Bradley and Jenkison, 1973; Madrid et al., 1975; Montgomery and Swenarchuk, 1977). As mentioned above, another protein like merosin is secreted by non-myogenic cells, which stabilizes the muscle fibers.

Proteins of interest to be expressed as restored structural proteins:

Dystrophin:

Myogenic cells may be transplanted to restore the expression of dystrophic *mdx* dystrophic mice The *mdx* mice are an animal model of Duchenne muscular dystrophy (Bulfield et al. 1984). As the human patients, this animal model lacks dystrophin due to a mutation of that gene located in the X-chromosome (Sicinski et al. 1989). The

absence of this protein under the sarcolemma lead to muscle fiber degeneration and to eventual muscle weakness (Pastoret and Sebille, 1995). The development of this disease may be prevented by transplanting normal myoblasts which restore the expression of dystrophin in the muscle fibers.

Merosin:

The *dy/dy* dystrophic mice are an excellent model of Congenital Muscular Dystrophy (Arahata et al. 1993; Sunada et al. 1994, 1995; Xu et al. 1994 a,b). The expression of merosin can be eventually restored by the transplantation in the muscle of *dy/dy* non-myogenic cells. Since this protein is present in the extracellular matrix it has to be secreted by the cells injected either directly in the muscles or injected in the blood and migrating in the muscles or in any other affected tissues, e.g., the peripheral nerves in the case of the *dy/dy* mice to restore the expression of merosin in the extracellular matrix surrounding the muscle fibers. The presence of merosin in the extracellular matrix stabilizes the muscle fibers and helps recovering a normal muscle function while the presence of merosin near the nerve could restore a normal propagation of action potentials.

Others:

Another examples of proteins of interest that can be expressed in transplanted myoblasts or other cell types exist. For example, the cytoplasmic restoration of glucose-6-phosphate dehydrogenase has been described in stable hybrid myotubes (Sansone et al.,1993; Meola et al.,1993). Therefore, the present invention applies at least to different types of myopathies of a genetic origin or other origins. Since other types of cells (neurons, islets, hepatocytes, macrophages and neutrophils) may be transplanted to cure diseases like Huntington, Parkinson diseases, diabetes and liver diseases, it will be readily appreciated that the present invention applies to all types of diseases that may be cured by transplanting healthy donor cells.

The above results show that it is possible to obtain proliferation of donor cells by way of a conditional immortalization and that such cells are suitable for transplantation and for restoring the function of a tissue by expressing a structural functional protein or a secreted protein.

EXAMPLE 6

Transplantation of myoblasts obtained by the genetic modification of fibroblasts

Transplantation of normal myoblasts into dystrophic muscles is a potential treatment for Duchenne muscular dystrophy (DMD). However, the success of such grafts is limited by the immune system responses. To avoid rejection problems, autologous transplantation of the patient's corrected myoblasts has been proposed. Regretfully, the low proliferative capacity of DMD myoblasts in culture (due to their premature senescence) limits such procedure. On the other hand, modification of dermal fibroblasts leading to the myogenic pathway using a master regulatory gene for

myogenesis is an interesting alternative approach. In this study, the retrovirally encoded MyoD1 cDNA was introduced in dermal fibroblasts of TnI LacZ mice to provoke their conversion into myoblast-like cells. *In vitro* and *in vivo* assays were done and the results were compared to those obtained with uninfected fibroblasts and myoblasts. Some MyoD1-expressing fibroblasts were able to fuse and to express β-galactosidase (under the transcriptionnal control of the Troponin I promoter), dystrophin and desmin *in vitro*. Thirty days following implantation of these modified fibroblasts in muscles of mdx mice, an average of 7 β-gal+/Dysmuscle fibers were observed. No β-gal+ fibers were observed after the transplantation of uninfected fibroblasts. Our results indicate that the successful implantation of myoblasts obtained from genetically modified fibroblasts is indeed feasible. However, the *in vitro* conversion rate and the in viro fusion of genetically modified fibroblasts must be largely increased to consider this approach as a potential therapy for DMD and other myopathies.

Duchenne muscular dystrophy (DMD) is an X-linked recessive disease characterized by the absence of dystrophin, a protein located beneath the sarcolemma of muscle fibers. Transplantation of normal myoblasts into dystrophic muscles is a potential therapy to compensate the lack of dystrophin expression in muscles of DMD patients. However, poor results were obtained in clinical trials involving the transplantation of myoblasts from healthy donors in the muscles of dystrophic patients. This limited success was in part due to specific and non specific immune reactions which destroy the injected cells and the hybrid fibers formed by the fusion of donor myoblasts with host fibers. This rejection could probably be overcame by immunosuppressive treatments as it was previously done in animals with FK506 or by grafting the patient's own myogenic cells after the introduction of the dystrophin gene. However, long-term use of immunosuppressors like FK506 or cyclosporine are known to cause important side-effects like nephrotoxicity, neurotoxicity and higher risks of infections. Moreover, myoblasts from DMD boys older than 4 years old have a low proliferative capacity and enter the senescence pathway earlier than normal myoblasts, limiting the autologous transplantation of DMD myoblasts engineered to carry the dystrophin gene.

Skeletal muscles are derived from the dorsal portion of somites, the dermomyotome, which is the source of muscle precursor cells and dermal fibroblasts of the dorsal region of the body. Proliferating myoblasts migrate from the dorsomedial region of the dermomyotome to form the myotome which will become the first differentiated skeletal muscle formed in the embryo. Mononuclear myoblasts, the precursors of skeletal muscle fibers, differentiate into myocytes under the control of a group of myogenic regulatory genes. Those myocytes fuse together to form multinucleated myotubes.

MyoD1 is a master regulatory gene for skeletal myogenesis. The product of this gene, a nuclear phosphoprotein, contains a basic-helix-loop-helix motif and a c-myc homology domain in a 68-amino acids region. This latter region is both sufficient and necessary to engage a variety of non-muscle cells into the myogenic lineage. MyoD is a DNA-binding protein that binds to the enhancer sequences of a number of muscle specific genes. Furthermore, it has been shown to activate its own transcription. This autoactivation could provide a positive feedback loop to keep cells engaged in the myogenic pathway following MyoD activation or a control mechanism to increase MyoD levels once the gene has been activated.

Other transcription factors of the MyoD family have also been identified: myogenin, myf-5 and herculin. All of these factors show a large degree of homology with the MyoD1 gene, but in at least some cases MyoD1 alone has been shown to activate the muscie-program in a variety of differentiated cell lines.

The introduction in dermal fibroblasts of the retrovirally encoded MyoD1 gene has been shown to convert these cells to myoblasts under specific conditions. In primary dermal fibroblasts, MyoD expression is repressed by trans-acting factors. The forced expression of this gene with a retroviral vector could down-regulate the endogenous program of these non-myogenic cells and permit the conversion to myogenic program. The use of dermal fibroblasts to eventually complement the dystrophin deficiency into dystrophic muscle is interesting because these cells are not functionally compromised by DMD and thus retain a high proliferative potential. Furthermore, the formation of normal muscle fibers in DMD patients could result from the graft of their own fibroblasts transduced with a myogenesis regulatory gene, an hypothesis first proposed by Tapscott et al.

We confirm that mouse dermal fibroblasts can be induced to express muscle markers, are able to form myotubes *in vitro* and fuse with host muscle fibers *in vivo* once infected with the retroviral vector containing MyoD1 cDNA.

MATERIALS AND METHODS

Dermal Fibroblast Culture

Transgenic TnI LacZ1/29 mice (generous gift from Dr K. E. Hasting, Mc Gill University, Montreal, Canada) contain the β-galactosidase cDNA under the control of the quail fast troponin I promoter. Primary fibroblast cultures were established from the dermis of newborn transgenic mice identified by the X-gal histochemical staining (Boehringer Mannheim Canada, Laval, Can.) of a small muscle biopsy. Dermal fibroblasts were isolated with a modified technique described elsewhere (32). Precisely, the skin of hind limbs was minced into 0,5 cm2 pieces and then digested in 0,25% trypsin (GIBCO BRL, Canada) overnight at 4°C. This treatment permits the complete dissociation of the epidermis from the dermis. After cutting into small pieces, the dermis was incubated with collagenase (0,1% (w/v); Sigma, St-Louis, MO,USA)

during 90 minutes at 37°C. Fibroblasts were washed twice and grown in Dulbecco's modified Eagle's medium with high glucose and with 10% fetal calf serum. In order to establish the fibroblasts as the predominant cell type in the culture, the cells were passaged 6 times before each experiment.

Retrovirus Production and *in vitro* Infection

The retroviral vector pLMDSN containing the MyoD coding region under the MoMLV LTR promoter and a neomycin resistance gene under the SV 40 early promoter was used to create the amphotrophic retrovirus PA317 LMDSN producing cell lines (generously donated by S. J. Tapscott from the Hutchinson Cancer Research Center). These PA317 LMDSN cells were grown in Dulbecco's modified Eagle's medium with high glucose and with 10% fetal calf serum. The culture supernatant was harvested after an overnight exposure to a confluent layer of PA317 LMDSN and stored at -70°C.

After six passages in culture, the myogenicity of the dermal fibroblast cultures was assessed by desmin immunostaining. These cells were then infected by three overnight exposures to the medium containing the LMDSN virus in the presence of polybrene (8 Hg/mL). Selection was done using G418 (600 1lg/mL).

in vitro Assay

Fusion test. Infected fibroblasts were grown to confluence on Labtek slides (Nunc) in DMEM high glucose (GIBCO BRL, Canada) containing 10'c horse serum. These conditions were maintained for 10 days to allow sufficient cell fusion. Non-infected dermal fibroblasts were used as control.

X-Gal staining. Cells were fixed on Labtek slides with glutaraldehyde 0,25" for 3 min. After 3 washes with PBS, cells were incubated overnight in the dark, at room temperature, with a X-Gal solution (3 mM X-Gal of potassium ferrocyanide, 3 mM of potassium ferricyanide, 0,1 mM of $MgCl_2$ and 0,4 mM of X-Gal in dimethyl formamide; Boehringer Mannheim Canada, Laval, Can.). Cell preparations were mounted in PBS-glycerol.

Desmin immunocytolluorescence. Fibroblast cultures were first fixed with 95'7c ethanol for 15 minutes and permeabilized with 5'7c Tnton X-100 for 25 minutes. Desmin was then detected with the monoclonal DE-R-11 antibody (1:40; Dako, Copenhagen, Denmark) followed by a biotinylated anti-mouse antibody (1:250; Dako) and Streptavidin-Cy3 (1:700; Sigma).

Dystrophin and MyoD1 immunocytochemistries. Dystrophin was detected in the infected fibroblast cultures with a polyclonal rabbit antibody that recognizes the dystrophin COOH-terminal portion of many species (generous gift from Dr. J. S. Chamberlain). The cells were first fixed in 95% ethanol for 15 minutes and permeabilized with 0,5'7s Triton X-100 for 15 minutes. Fixed cultures were then incubated with the anti-dystrophin antibody (1:1000) followed by a biotinylated

anti-rabbit antibody (1:250) and Streptavidin-Cy3 (1:700).

MyoD1 staining was done as previously described.

in vivo Assay

Cell transplantation. For transplantation, the G418-selection step was omitted because myoblasts were found to have a reduced capacity to fuse and proliferate after such a selection (data not shown). Dermal fibroblasts were transplanted in the Tibialis anterior (TA) muscle of C57BL/10 Sn J mdx/mdx mice (Jackson Laboratory, Bar Harbor, ME, USA). Three days before transplantation, TA muscles were irradiated (20 Gy) to block the proliferation of host myoblasts. Furthermore, the day before transplantation, TA muscles were exposed and injected with notexin venom (5 1lg/ml) to trigger muscle fiber degeneration. For transplantation, the skin of hind limbs was cut to expose the TA muscle and roughly 40 sites were injected with a total of 4 x 106 transfected fibroblasts using a micropipette (Drummond Co., Broomal, PA, USA). Non-infected dermal fibroblasts were grafted as negative and TnI LacZ myoblasts were transplanted as positive controls. The skin was sutured using 6.0 silk (Johnson & Johnson, Peterborough, Ont., Can.).

Following transplantation, mice were immunosuppressed with a daily dose of FK506 (2,5 mg/kg i.m.; Fujisawa Pharmaceutical Co. Ltd. Osaka, Japan).

Mouse sacrifice. Mice were sacrified 30 days after transplantation under deep anesthesia (0,1 ml of a solution containing ketamine (10 mg/ml) and xylazine (10 mg/ml)). After perfusion with a NaCl 0,9% solution containing heparin (2 IU/ml) (Organon Teknika, Toronto, Ont., Can.), muscles were removed and placed in PBS with 30% (W/V) sucrose overnight at 4°C. Muscles were then embedded in cryomatrix (Shandon, Pittsburgh, PA, USA) and frozen in liquid nitrogen. Muscles were cut in 10 Hm thick sections using a cryostat microtome and picked up on gelatin coated slides.

X-Gal staining. Muscle sections were fixed in glutaraldehyde 0,25% for 3 min. After 3 washes, slides were incubated overnight at room temperature with X-Gal solution and mounted in PBS-glycerol.

Dystrophin immunohistochemistry. The endogenous peroxidase activity in muscle sections was first blocked using 1% hydrogen peroxide. Nonspecific binding sites were blocked with 10% calf serum. Muscle sections were then incubated with the anti-dystrophin antibody (1:1000) followed by a biotinylated anti-rabbit antibody (1:250) and Streptavidin-Cy3 (1:700).

Hematoxylin-eosin staining. Muscle cross sections were briefly incubated with hematoxylin and with eosin, dehydrated with successive washes in 70% ethanol, 100% ethanol and toluene. Slides were mounted with permount.

RESULTS

Characterization of Infected Dermal Fibroblasts in Culture

Dermal fibroblasts were grown in culture for six passages to avoid myogenic

cell contamination prior to *in vitro* and assays. The purity of the fibroblast cultures was confirmed by immunostaining using antibody to the intermediate filament protein desmin. All cultures were found negative for desmin. Moreover, cells failed to fuse and form myotubes when cultured for 10 days in a differentiation medium containing 10% horse serum. These cells were mononuclear with the usual fibroblast-like morphology.

Cells were then infected with the LMDSN amphotrophic retrovirus and selected in G418 for 10 days. We observed that 30% of the infected fibroblasts survived G418 selection. The expression of muscle-specific proteins and morphological characteristics in MyoD-infected and control cells were studied *in vitro*. Three days after the infection, cultures began to show morphological changes such as cell elongation (data not shown). These morphological changes were not observed in the controls. Some infected cells expressed desmin and MyoD1 proteins (Fig. 8a, b). These cells were also able to fuse in differentiation medium and form multinucleated myotubes expressing β-gal and dystrophin (Fig. 8c, d). No β-Gal (Fig. 8e), desmin, dystrophin and MyoD 1 staining was detected in uninfected dermal fibroblasts.

Transplantation of Dermal Fibroblasts

Irradiated TA muscles of mdx mice were transplanted either with $4X10^6$ uninfected dermal fibroblasts or with $4X10^6$ LMDSN-infected fibroblasts. The infected fibroblasts were not selected with G418 prior to their transplantation to preserve their proliferative and myogenic capacities. Some mice were grafted with $4X10^6$ myoblasts as positive controls. Thirty days following transplantation, mice were perfused and the engrafted muscles were frozen and cut in 10 Hm thick sections for subsequent analysis.

Thirty days post-transplantation, MyoD1-infected fibroblasts fused poorly with host's fibers, producing an average of 7 small β-galactosidase positive fibers (Fig. 8g) while TnI LacZ myoblasts formed abundant fibers expressing β-Gal (Fig. 8h). These 7 fibers were dystrophin negative (Fig. 8i). Many β-Gal negative but dystrophin-positive fibers were seen throughout the mouse muscles (data not shown). These were more likely revertant fibers (34, 35) than hybrid fibers coming from the fusion of modified fibroblasts since they were β-gal negative. Interestingly, the β-galactosidase positive fibers had a centrally located nucleus indicating a recent regenerative process: a phenomenon not observed in revertant fibers. Except for the overall reduced efficiency, muscle fibers originating from MyoD converted fibroblasts were indistinguishable from those derived from the TnI LacZ myoblasts. No fibers expressing β-galactosidase were found in muscles injected with dermal fibroblasts (Fig. 8f) although some of them expressed dystrophin. As described before, we consider those fibers as revertants.

Hematoxylin-eosin coloration was also done to stain the fibroblast nuclei and the connective tissue present in the muscle sections. This staining revealed a high

proportion of connective tissue in the muscles injected with both MyoD-infected and normal fibroblasts (Fig. 8j). This high proportion of connective tissue was not present in muscles transplanted with myoblasts.

DISCUSSION

MyoD1 is a master regulatory gene for skeletal myogenesis. Forced expression of this transcription factor in a variety of differentiated cell types allow their conversion to myoblasts by the activation of muscle differentiation markers.

In the present study, the myogenic potential of MyoD1 was used to convert dermal fibroblasts to myoblasts, as previously reported by Weintraub. Fibroblasts were infected with a retroviral vector containing the coding section of the MyoD1 gene. Differentiation of some cells was observed and muscle-specific proteins were detected, even if the transformation rate was low. Cell fusion also occurred.

The high proliferative capacity and the possibility of autologous transplantation render the use of genetically modified fibroblasts very attractive as an alternative approach to the myoblast transfer therapy. Previous studies on myoblast transplantation into mdx mouse muscle have shown the formation of numerous dystrophin-positive fibers in the injected muscles. In the present study, control transplantation of normal TnI LacZ muscle primary cultures have also led to numerous β-gal expressing fibers. However, injection of MyoD1-infected fibroblasts resulted in a surprisingly low amount of β-gal+ fibers in mdx muscles. The presence of β-galactosidase in these fibers indicate that they originated from the implanted cells which encode the β-galactosidase bacterial enzyme under a muscle specific promoter. These results are in accordance with those recently obtained by Lattanzi et al. following the injection of adeno-MyoD-converted fibroblasts in the TA muscle of SCID/bg mice. In that study, despite an infection of the fibroblasts with a high MOI (2000), only a few muscle fibers were formed by the infected fibroblasts.

The difference in muscle fiber formation efficiency between myoblasts and MyoD-infected fibroblasts could be explained in part by 1) the low percentage (30%) of fibroblasts infected with the retrovirus encoding MyoD and/or by 2) a premature growth arrest in MyoD-expressing fibroblasts early after infection which could decrease the amount of myogenic cells. A third possible explanation could be that only a small portion of MyoD-expressing fibroblasts were efficiently transformed into the myogenic lineage as previously described. However, no muscle fibers expressing β-gal were seen in muscles transplanted with non-infected dermal fibroblasts. A previous report by Gibson et al. has shown that dermal fibroblasts can give rise to muscle fibers when injected into skeletal muscle of mdx mice. In that report, the fusion of fibroblasts with myogenic cells was sufficient to induce their conversion to the myogenic pathway. Salvatori et al. have also demonstrated that the *in vivo* myogenic conversion of 10T1/2 cells, as well as primary skin fibroblasts, requires close cell-cell interaction but not

necessarily cell fusion with myogenic cells. This phenomenon was related to the embryological origin of fibroblasts. Difference between these results and those obtained in our experiments may be explained by the different lines of fibroblasts used for transformation.

No multinucleated myotubes formed by the fusion of MyoD-converted fibroblasts expressed dystrophin *in vivo* even if some fibers are expressing β-galactosidase. It could be explained by a lower expression of dystrophin in hybrid fibers formed by the fusion of transformed fibroblasts compared to the normal dystrophin expression levels seen when myoblasts are injected. In our experiments, the β-gal+ fibers may express dystrophin too weakly to be visualized by immunostaining. To permit the formation of dystrophin positive fibers by dystrophic MyoD-converted fibroblasts, mini-dystrophin gene might be inserted in the same vector carrying the MyoD gene. Thus, the reduced myogenicity of myoblast pool and the dystrophin deficiency characteristic of DMD may be corrected. Recent work in our laboratory confirmed that the transplantation in SCID mouse muscles of mdx and DMD myoblasts transduced *ex vivo* with a dystrophin minigene led to the formation of numerous Dys+ fibers.

We have observed connective tissue in sections obtained from muscles engrafted with normal and infected dermal fibroblasts. In comparison, the connective tissue was less abundant in muscles transplanted with myoblasts. It is well known that fibroblasts are the major cellular constituent of the dermis, a layer of connective tissue. The role of dermal fibroblasts is to synthesize collagen. In our experiments, one problem encountered is that roughly 30% of fibroblasts were converted to myoblasts following infections with the LMDSN retrovirus. It seems that following their transplantation, MyoD-converted fibroblasts lost their proliferative capacity, exited the cell cycle and entered the myogenic differentiation pathway early. Therefore, the non-converted fibroblasts still present in the culture divided more rapidly than transformed cells and produced connective tissue in the grafted muscle.

Very few muscle fibers were formed by the genetically modified fibroblasts probably because the conversion to the myogenic lineage necessarily implies the down-regulation of the endogenous differentiation program of the infected cells. There is possibly a coexistence of this endogenous developmental program and the myogenic program imposed by MyoD. It is possible that MyoD cannot turn off the endogenous program of all infected fibroblasts because they are already differentiated cells. It implies that the presence of MyoD is not necessarily sufficient to activate a strong skeletal muscle program *in vivo*. Another explanation for the small number of muscle fibers formed, is the shut-off of the retroviral promoter. Palmer et al. have reported that transplanted skin fibroblasts genetically engineered to carry the human adenosine deaminase gene persisted long after transplantation but the retrovirally

transferred gene was gradually inactivated, to reach undetectable level of the protein after 1 month. It is therefore possible that the LTR promoter of the MyoDI transgene was shut-off *in vivo*. However, the MyoDI produced by the transgene supposedly activates the natural MyoD1 promoter of the endogenous gene. Therefore, it is possible that when MyoD1 is initially produced in fibroblasts by the transgene, the cells remain committed to a myogenic differentiation even if the retroviral promoter is shut-off. So, a promoter shut-off may not be the only or the best theory to account for the poor success of the transplantation of genetically modified fibroblasts.

Another possibility to obtain a higher myogenic conversion rate is the injection of a replication-defective adenovirus containing MyoD following the graft of dermal fibroblasts. Murry et al. have shown that the *in vivo* injection of a high dose adenovirus encoding MyoD in cardiac granulation of an healing heart induced the differentiation of these cells into skeletal muscle.

A recent study reported that genetically marked bone marrow-derived cells migrate into areas of muscle degeneration, undergo myogenic differentiation and supply some satellite cells necessary for the formation of new muscle fibers. This approach was not also very effective, since only a small amount of muscle fibers were formed following directly intramuscular injection of the genetically modified bone marrow cells. The MyoD-retroviral vector may also be used to infect mesodermal progenitors, which are less differentiated than fibroblasts, providing an alternative strategy to the myogenic conversion of dermal fibroblasts.

Infection of fibroblasts with other members of the MyoD family, such as myogenin, could perhaps be tried to improve the myogenic conversion rate at the onset of muscle differentiation.

Thus, the *in vitro* myogenic conversion rate and the *in vivo* fusion of dermal fibroblasts transformed with the coding section of the MyoD1 gene must be greatly improved to enhance the formation of normal muscle fibers in mdx mouse muscles. The use of genetically modified fibroblasts could then be considered as potential therapy to increase the formation of skeletal muscle at the onset of the muscle weakness characteristics of Duchenne muscular dystrophy and other myopathies.

What has been produced with $MyoD_1$ gene expression may be reproduced with other myogenic genes.

A list of myogenic genes is provided below with their respective Gene Bank accession number:

<u>MyoD Gene Family</u>

Herculin	*mouse*	*M30499*
MyoD	*mouse*	*M84918*

MyoD	*rat*	*M84176*
Myogenin	*rat*	*M24393*
MyoD	*chicken*	*L34006*
MyoD	*human*	*X56677*
MyoD	*human*	*AC004736*
MyoD	*mouse*	*AI119463*
Myogenin	*rat*	*AF054894*
Myogenin	*mouse*	*AA770807*
MRF-4	*chicken*	*D10599*
Myogenin	*mouse*	*D90156*
MYF-4	*human*	*X17651*
Myogenin	*mouse*	*X15784*
Myogenin	*rat*	*M24393*
MYF-5	*human*	*X14894*
MRF-4	*rat*	*M84685*
MRF-4	*rat*	*M27151*
MYF-6	*human*	*X52011*
MYF-6	*mouse*	*X59060*

More genes may be discovered and added to this list which should not therefore by considered as restrictive. For each gene member being of non-human origin, the human counterpart certainly exists and it would be routine experimentation of obtain the human gene. If myoblasts are transplanted as xenografts, it is possible to insert a gene of different species, while results in a transformed donor myoblasts to be transplanted in another species. A suitable immuno suppressive therapy (specific or not) may be undertaken to support graft survival.

EXAMPLE 7

Treatment of Duchenne/Becker muscular dystrophies by specific mutagenesis of the utrophin promoter.

Duchenne and Becker muscular dystrophies are due to mutation of the dystrophin gene (Hoffman et al 1987). The mutation leads to the absence of a functional dystrophin molecule in the case of the Duchenne dystrophy and to a defective dystrophin in the case of Becker dystrophy (Arahata et al. 1988; Sugita et al. 1988; Worton et al. 1988; Zubrzycka-Gaarn et al 1988).

Utrophin (also called dystrophin related protein, DRP) is a protein which is very

similar to the dystrophin protein (Campbell 1991; Pons et al. 1991, 1993; Schofield et al 1993). The group of Kay Davies has recently demonstrated that the expression of utrophin could compensate for the absence of dystrophin in the mdx dystrophic mice (Tinsley et al. 1996).

Indeed in transgenic mdx mice expressing the utrophin gene have normal muscles and no increase of the creatine kinase (CK) activity in their serum.

Utrophin gene is expressed only at the neuromuscular junction in the adult muscle fibers (Gramolini et al. 1997). Utrophin can bind with the dystrophin associated glycoprotein complex (Mizuno et al. 1994). If this protein would be expressed throughout the muscle fibers, the results of Davies group indicates that this would reduce the vulnerability of muscle fibers of dystrophic animals or humans.

In the promoter of the utrophin there is an N box which is responsible for the specific synaptic expression (Dennis et al. 1996). Similar N boxes conferring specific synaptic expression have also been described for the d and the e subunit of the Acetylchloline Receptor genes (Koike et al. 1995; Duclert et al. 1996). A single base mutation of the second base of that N box suppress the specific synaptic expression and the e subunit is expressed everywhere on the muscle fiber (Duclert et al. 1996).

It is therefore possible to speculate that a single base mutation of the N box present in the utrophin promoter will permit to have an ubiquitous expression of this protein in the muscle fiber.

A method to make a single base pair mutation has been recently described by Cole-Strauss et al. 1996. This method makes use of an oligonucleotide made of DNA and RNA.

Myoblasts (also called satellite cells) are the cells which form muscle fibers by their fusion (Trupin et al. 1982). Following transplantation in a muscle, myoblasts fuse with the host muscle fibers. The muscle fibers formed by the fusion of myoblasts express the genes present in the donor myoblasts (Bartlett et al. 1990, 1995; Huard et al. 1991, 1993, 1995; Kinoshita et al. 1994 a, b; Partridge et al. 1989; Vilquin et al. 1995; Yao and Kurachi1992; Zatloukal et al. 1994).

It is therefore possible to treat Duchenne and Becker muscular dystrophies by mutating one or several nucleotides in the N box of the utrophin promoter. Such a mutation would permit a non synaptic expression of utrophin which would compensate for the absence of dystrophin. This mutation can be done *in vitro* by mutating myoblasts before their transplantation or *in vivo* by injection of the mutating oligonucleotide in the muscle or in the blood circulation. To effect such a mutation, 1 to 6 nucleotides of the N box of the utrophin promoter are mutated. Preferably, the second nucleotide of the N box of the utrophin promoter is changed from a C to a T when oligonucleotides are used to effect such a mutation they have the following two sequences:

5'GGC TGA TCT TCC AGA ACA AAG TTG CTT TTg caa cuu ugu TCT GGa aga uca gcc GCG CGT TTT CGC GC3'

5'GCA ACT TTG TTC TGG AAG ATC AGC CTT TTg gcu gau cuu CCA GAa caa agu ugc GCG CGT TTT CGC GC3'

where the small characters represent RNA and the large characters are DNA nucleotides.

An *in vivo* mutation is achieved by injecting an oligonucleotide in the muscle or in the blood circulation. The mutation of the utrophin promoter can also be done in myoblasts *in vitro*. The myoblasts were obtained from a donor, and may be the patient's own myoblasts. The same oligonucleotides as above may be used. The mutation of the utrophin promoter is also done in fibroblasts *in vitro*. The fibroblasts were obtained from a donor. These may be the patient's own fibroblasts. In that case also, the same oligonucleotide are used.

The fibroblasts are converted in myoblasts before their transplantation in the patient muscles, by inserting a myogenic gene. The myogenic gene is that of MyoD, myogenin or Myf5.

The mutation of the utrophin promoter is also done in stem cells *in vitro*. The stem cells were obtained from a donor. They may be patient's own stem cells. The same oligonucleotides as above are used.

The stem cells are converted in myoblasts before their transplantation in the patient muscles by inserting a myogenic gene. The myogenic gene is that of MyoD, myogenin or Myf5.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

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WHAT IS CLAIMED IS:

1. A composition for increasing the survival of transplanted cells upon their transplantation or injection into a host, said composition comprising an anti-inflammatory amount of an anti-inflammatory agent which interferes with the recruitment, the binding, or the activation of pro-inflammatory cells of the host toward said cells, so as to prevent the destruction of said transplanted cells by said host, with the proviso that said composition does not essentially consist of an anti-LFA-1 antibody or anti-ICAM-1 antibody fragment, and a pharmaceutically acceptable vehicle.

2. A composition as defined in claim 1, which further comprises the transplanted cells.

3. A composition as defined in claim 2, wherein said transplanted cells are genetically engineered to express and secrete said anti-inflammatory agent.

4. A composition as defined in any one of claims 1 to 3, wherein said anti-inflammatory agent comprises one or more of the following: TGFbeta1, an inhibitor of oligosaccharide synthesis, a glucosidase, IL-10, vIL-10, IL-4, INFgamma, IL-2R, IL-1Ra, Fas-L, sCR1, a super oxide dismutase, a neutrophil inhibitory factor (NIF), a ligand binding in an antagonist fashion to LFA-1, MAC-1, ICAM-1, CD-18, CD-31, CD-50, E-selectin, P-selectin, TNFalpha, IL-1 and IL-8.

5. A composition as defined in claim 4, wherein said anti-inflammatory agent further comprises a ligand against LFA-1 or ICAM-1.

6. A composition as defined in claim 4 or 5, wherein said ligand is an antibody or an antibody lacking its constant region.

7. A composition as defined in any one of claims 1 to 6, which further comprises an effective amount of a metalloprotease or of an agent capable of inducing the production of metalloprotease by said transplanted cells, or both, whereby the transplanted cells are capable of spreading into a recipient tissue of the host and of fusing with the cells of the tissue of the host.

8. A composition as defined in claim 7, wherein the metalloprotease is one or more of the following: gelatinase A (MMP-2), gelatinase B (MMP-9), collagenase-1 (MMP-1), collagenase-2 (MMP-8), collagenase-3 (MMP-13), collagenase-4 (MMP-18), stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), stromelysin-3 (MMP-11),

metalloelastase (MMP-12), matrilysin (MMP-7), MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT2-MMP (MMP-16), MT3-MMP (MMP-16) and MT4-MMP (MMP-17).

9. A composition as defined in claim 8, wherein the metalloprotease is matrilysin (MMP-7).

10. A composition as defined in claim 7, wherein the inducing agent is concanavalin A or a phorbol ester.

11. A composition as defined in claim 7, 8, 9 or 10, which further comprises the transplanted cells.

12. A composition as defined in claim 11, wherein the transplanted cells are genetically engineered to express and secrete said metalloprotease.

13. A composition as defined in any one of claims 2 to 12, wherein said transplanted cells are grown *ex vivo* in the presence of a growth or a trophic factor.

14. A composition as defined in claim 13, wherein said growth or trophic factor is one or more of the following: insulin, insulin like growth factor type 1 (IGF-1), insulin like growth factor type II (IGF-II), basic fibroblast growth factor (bFGF, FGF-1, FGF-2, FGF-4, FGF-6), acidic FGF, keratinocyte growth factor (FGF-7), interleukine-1 (IL-1), interleukine-6 (IL-6), transforming growth factor alpha (TGFalpha), transforming growth factor beta1 (TGFbeta1), nerve growth factor (NGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF), hepatocyte growth factor1 scattor factor (HGF/SF), heparin binding epidermal growth factor-like growth factor (HB-EGF), tumor necrosis factor (TNFalpha) and transferin.

15. A composition as defined in claim 14 wherein said growth or trophic factor is bFGF.

16. A composition as defined in any one of claims 13 to 15, wherein said transplanted cells are genetically engineered to produce said trophic or growth factor.

17. A composition as defined in any one of claims 2 to 16, wherein said transplanted cells are genetically engineered to express a gene which is capable of triggering and maintaining proliferation of the transplanted cells *in vitro*, and which is incapable or made incapable of maintaining said proliferation of said transplanted cells upon their transplantation.

18. A composition as defined in claim 17, wherein said gene is a gene encoding a thermosensitive mutant of SV40 large T antigen, a c-myc gene or a telomerase gene.

19. A composition as defined in claim 18, wherein said gene encodes a thermosensitive mutant of SV40 large T antigen.

20. A composition as defined in claim 19, wherein said transplanted cells are grown *in vitro* at about 33°C.

21. A composition as defined in claim 17, 18, 19 or 20, wherein said gene is expressed under the control of an inducible promoter.

22. A composition as defined in claim 21, wherein said inducible promoter is H2K promoter which is inducible by IFNgamma, or is a promoter inducible by tetracycline, doxycycline, metallothionein, ecdysone or RU-486.

23. A composition as defined in any one of claims 2 to 22, wherein said transplanted cells are myoblasts.

24. A composition in any one of claims 2 to 22, wherein said transplanted cells are fibroblasts or mesenchymatous stem cells transformed into myoblasts by inserting therein and expressing a myogenic gene.

25. A composition as defined in claim 24, wherein said myogenic gene is one or more of myo-D1, myogenin, myf-5, MRF-4 and herculin.

26. A composition as defined in claim 25, wherein said myogenic gene is MyoD1.

27. A composition as defined in any one of claims 24 to 26, wherein said transplanted cells are genetically engineered to express a functional protein capable of restoring a physiological function depending on the action of said functional protein.

28. A composition as defined in claim 23, wherein said transplanted cells are genetically engineered to express a functional protein capable of restoring a physiological function depending on the action of said functional protein.

29. A composition as defined in claim 27 or 28, wherein said functional protein is selected from the group consisting of dystrophin, utrophin, a sarcoglycan, a dystroglycan, a syntrophin, a sarcospan, merosine or a metabolic enzyme, a

coagulation factor, an hormone or growth factor, myotonine kinase, tyrosine hydroxylase, a major histocompatibility complex (MHC) protein, arylsulfatase A and granulocyte colony stimulating factor (G-CSF).

30. A composition as defined in claim 29, wherein said functional protein is dystrophin.

31. A composition as defined in any one of claims 24 to 28 and 30, wherein said host is a patient suffering of Duchenne or Becker muscular dystrophy.

32. A composition as defined in claim 23, wherein said host is a patient suffering of Duchenne or Becker muscular dystrophy.

33. A composition as defined in claim 31 or 32, wherein said transplanted cells are genetically engineered to express utrophin as a dystrophin-like protein by mutating the utrophin gene promoter.

34. A composition as defined in of claim 33, wherein said mutation of the utrophin promoter is done in myoblasts *in vitro*.

35. A method of claim 33, wherein said mutation of the utrophin promoter is done in fibroblasts *in vitro*.

36. A composition as defined in of claim 33, wherein said mutation of the utrophin promoter is done in mesenchymatous stem cells *in vitro*.

37. A composition as defined in any one of claims 23, and 28 to 34, wherein said myoblasts are obtained from a donor.

38. A composition as defined in any one of claims 23, and 28 to 34, wherein said myoblasts are the host's own myoblasts.

39. A composition as defined in any one of claims 24 to 33, 35, and 36, wherein said fibroblasts or stem cells are obtained from a donor.

40. A composition as defined in any one of claims 24 to 33, 35, and 36, wherein said fibroblasts or stem cells are the patient's own fibroblasts or stem cells.

41. A composition as defined in any one of claims 33 to 40, wherein said mutation

is a mutation of the N box in the utrophin promoter.

42. A composition as defined in claim 41, wherein 1 to 6 nucleotides of the N box of the utrophin promoter are mutated.

43. A composition as defined in claim 42, wherein the second nucleotide of the N box of the utrophin promoter is changed from a C to a T.

44. A composition as defined in any one of claims 33 to 43, wherein said utrophin promoter is mutated by using an oligonucleotide.

45. A composition as defined in claim 44, wherein said oligonucleotide is made of DNA and RNA.

46. A composition as defined in claim 45, wherein said oligonucleotide to mutate the utrophin promoter has the following two sequences:

5'GGC TGA TCT TCC AGA ACA AAG TTG CTT TTg caa cuu ugu TCT GGa aga uca gcc GCG CGT TTT CGC GC3'
5'GCA ACT TTG TTC TGG AAG ATC AGC CTT TTg gcu gau cuu CCA GAa caa agu ugc GCG CGT TTT CGC GC3'
where the small characters represent RNA and the large characters are DNA nucleotides.

47. A composition as defined in any one of claims 33 to 36 and 41 to 46, wherein said mutation of the utrophin promoter is done *in vivo*.

48. A composition as defined in claim 47, wherein said mutation of the utrophin promoter is done *in vivo* by injecting the oligonucleotide in the muscle or in the blood circulation.

49. A composition as defined in any one of claims 2 to 6, wherein said host is affected by inflammatory disease such as arthritis or sporiasis.

50. A composition as defined in claim 23, 37 or 38 where the tissue is a cardiac or a skeletal muscle.

51. A composition as defined in any one of claims 1 to 50, wherein the cells are injected into the arterial or venous circulation of the host.

52. A composition as defined in claim 51, wherein said cells are genetically modified to express adhesion molecules to increase their binding to endothelial cells.

53. A composition as defined in claim 51 or 52, wherein said cells are genetically modified to express a metalloproteinase to improve their passage from the circulation to the tissue.

54. A composition as defined in any one of claims 1 to 6, wherein said transplanted cells are macrophages and neutrophils genetically engineered to produce a matrix protein and injected in the blood of said host.

55. A composition as defined in claim 50, wherein the tissue of the host is a heart, to prevent or treat heart insufficiency.

56. A composition as defined in any one of claims 1 to 55, which further comprises an immunosuppressive agent capable of rendering the host immunotolerant toward said transplanted cells.

57. A composition as defined in claim 56, wherein said transplanted cells are genetically modified to express said immunosuppresive agent.

58. A composition as defined in claim 56, wherein said immunosuppressive agent is FK506, cyclosporin, cyclophosphamide or rapamycin.

59. A composition as defined in anyone of claims 1 to 58, wherein said transplanted cells constitute a xenograft for said host.

60. A composition as defined in claim 59, wherein said xenograft is a porcine transplant for a human host.

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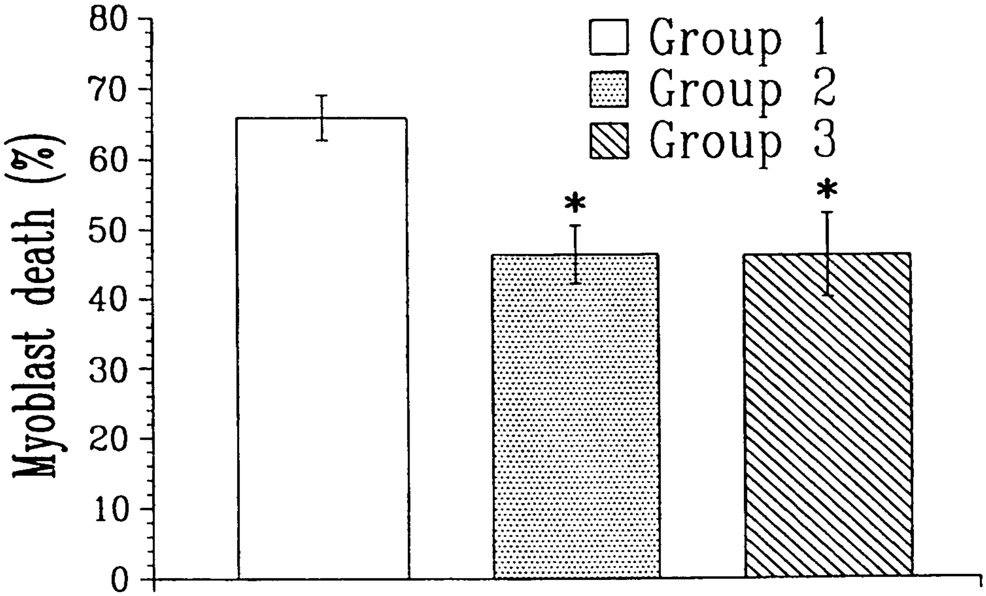


FIG. 1

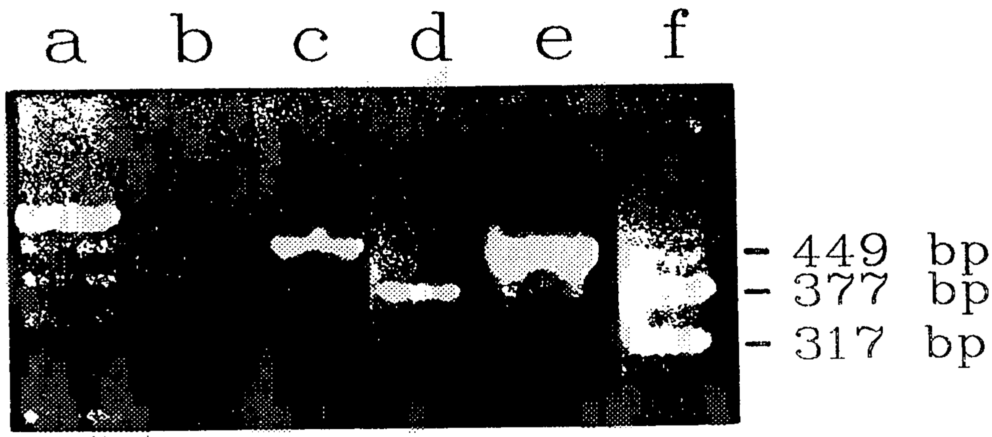


FIG. 2

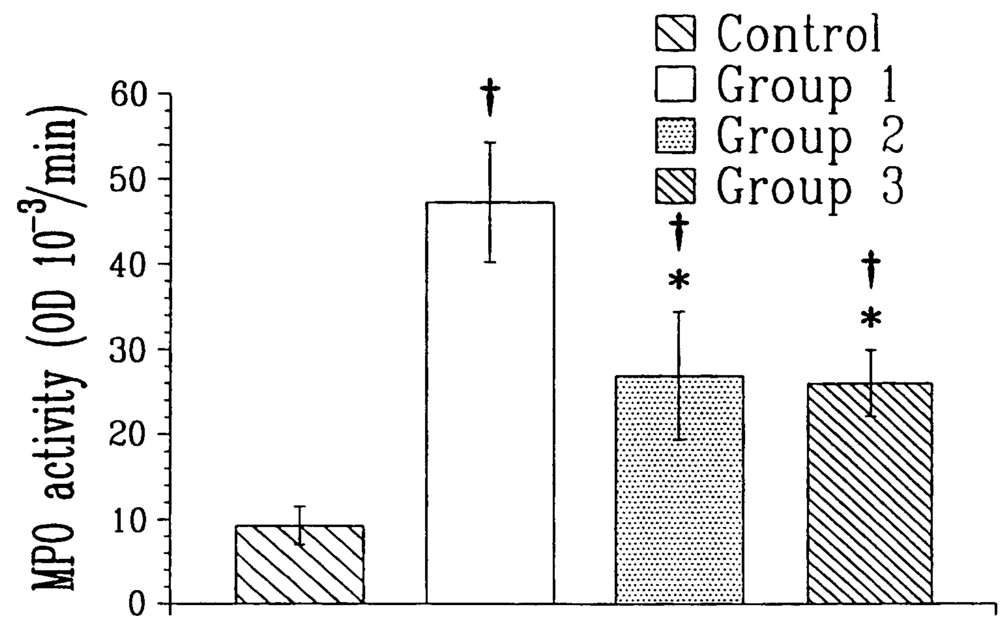

Control
Group 1
Group 2
Group 3
MPO activity (OD 10^{-3}/min)
0
10
20
30
40
50
60
†
*

FIG. 3

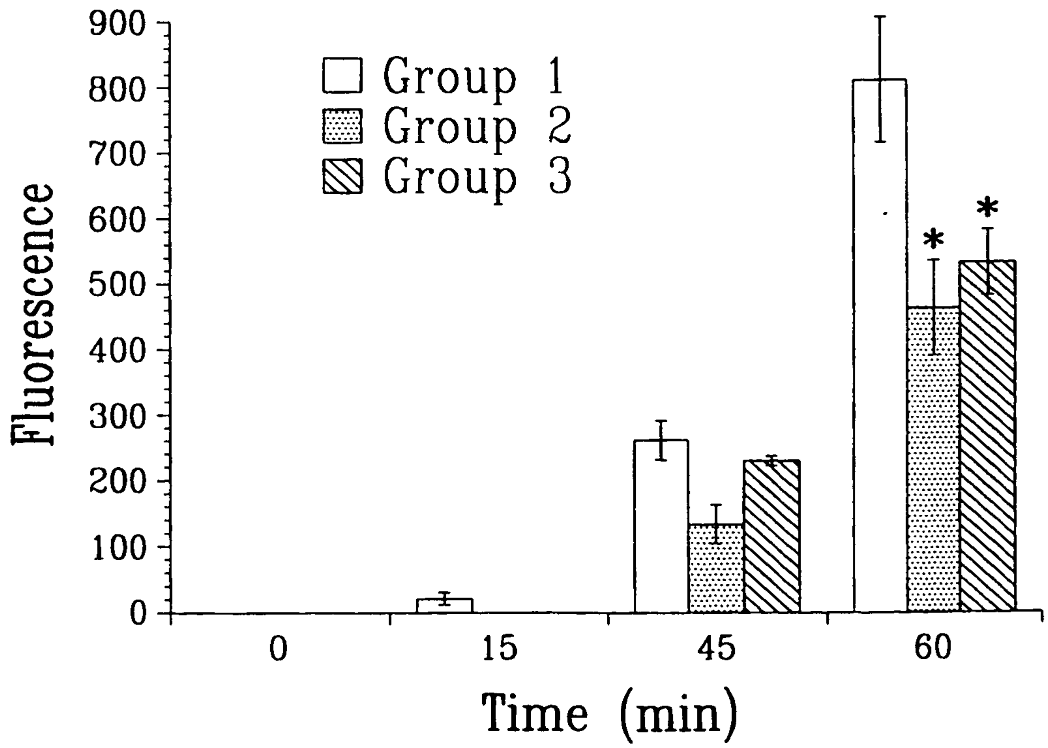

Group 1
Group 2
Group 3
Fluorescence
0
100
200
300
400
500
600
700
800
900
0
15
45
60
Time (min)
*

FIG. 4

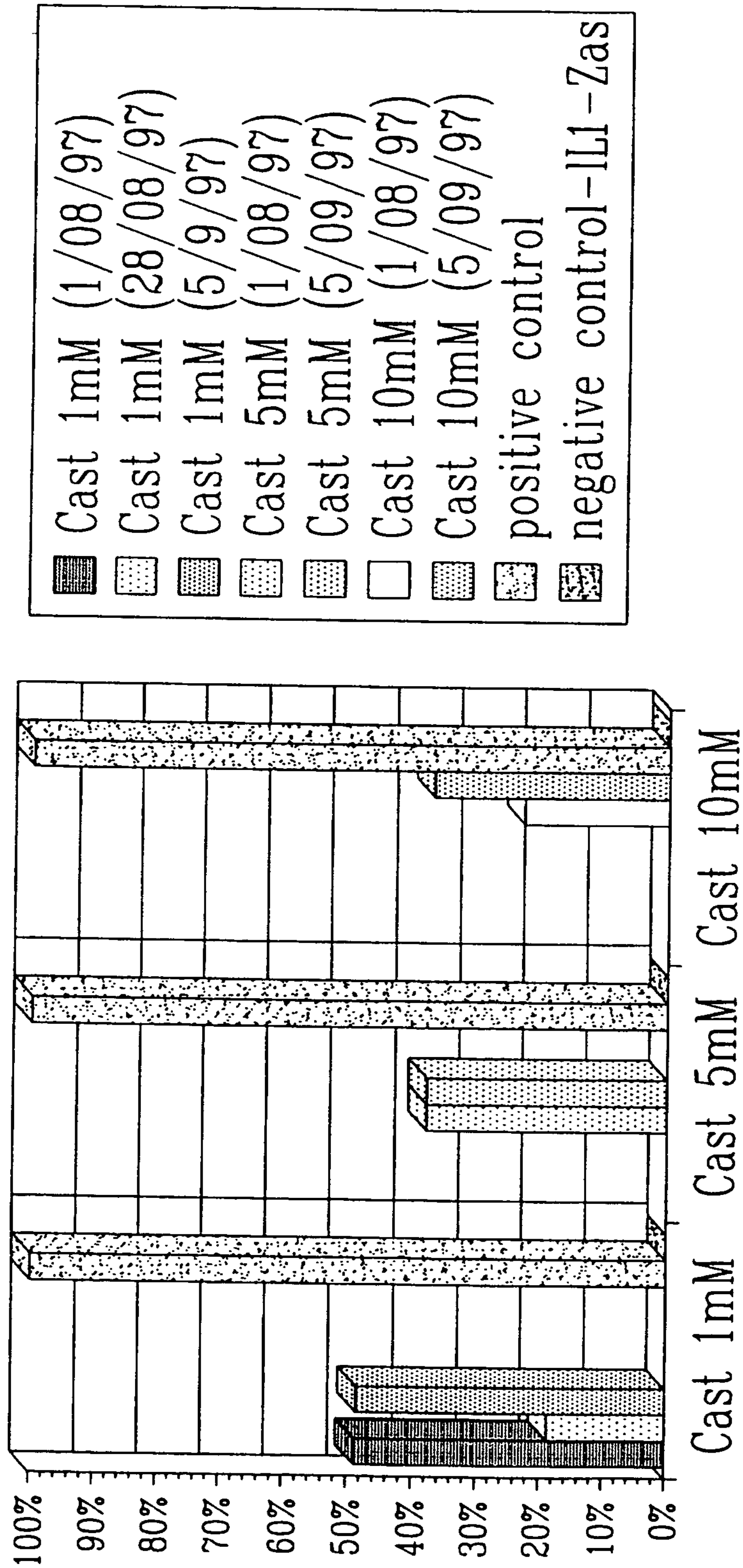
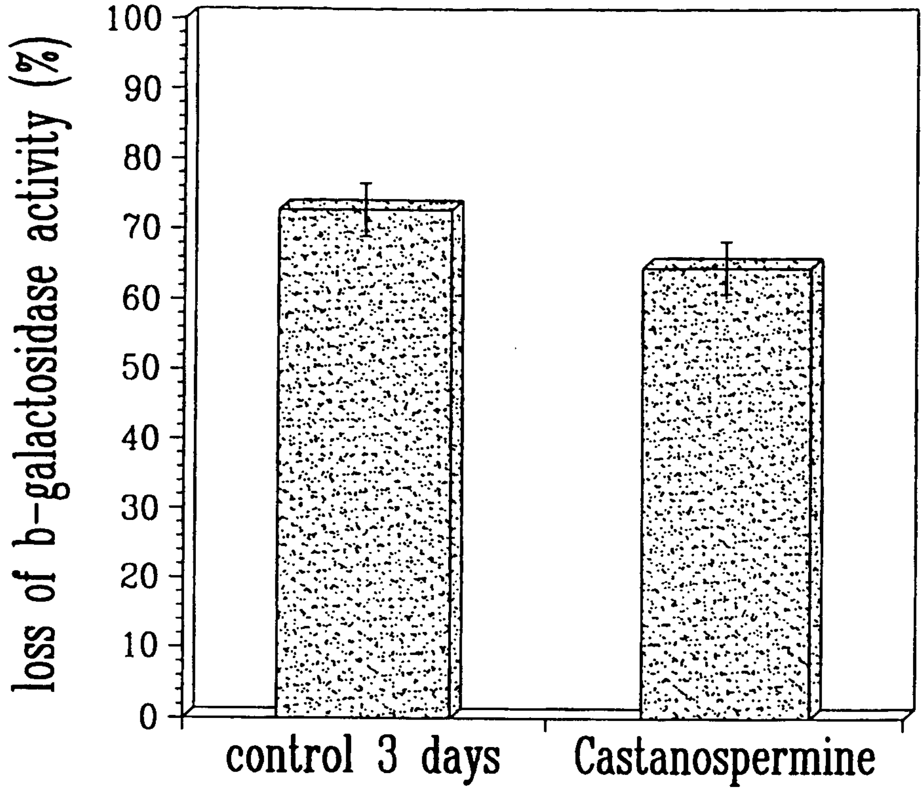
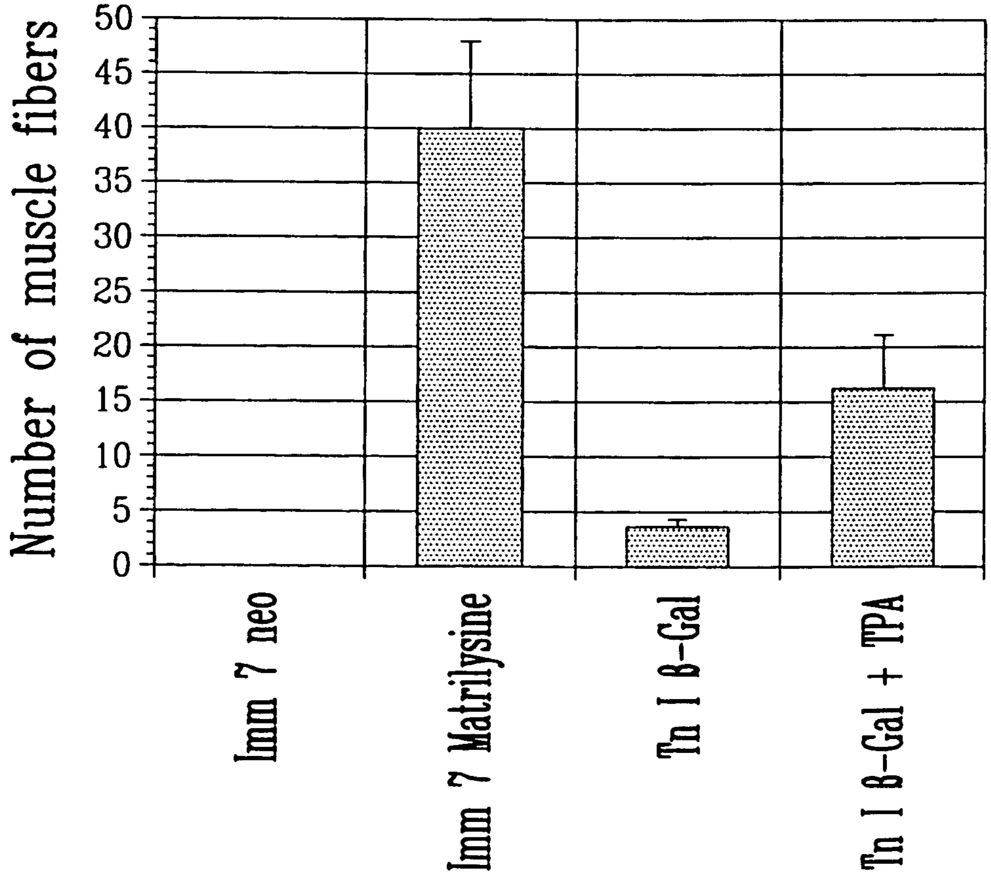

Cast 1mM (1/08/97)
Cast 1mM (28/08/97)
Cast 1mM (5/9/97)
Cast 5mM (1/08/97)
Cast 5mM (5/09/97)
Cast 10mM (1/08/97)
Cast 10mM (5/09/97)
positive control
negative control-IL1-Zas
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Cast 1mM
Cast 5mM
Cast 10mM

FIG. 5



loss of b-galactosidase activity (%)
100
90
80
70
60
50
40
30
20
10
0
control 3 days
Castanospermine

FIG. 6



Number of muscle fibers
50
45
40
35
30
25
20
15
10
5
0
Imm 7 neo
Imm 7 Matrilysine
Tn I ß-Gal
Tn I ß-Gal + TPA

FIG. 7

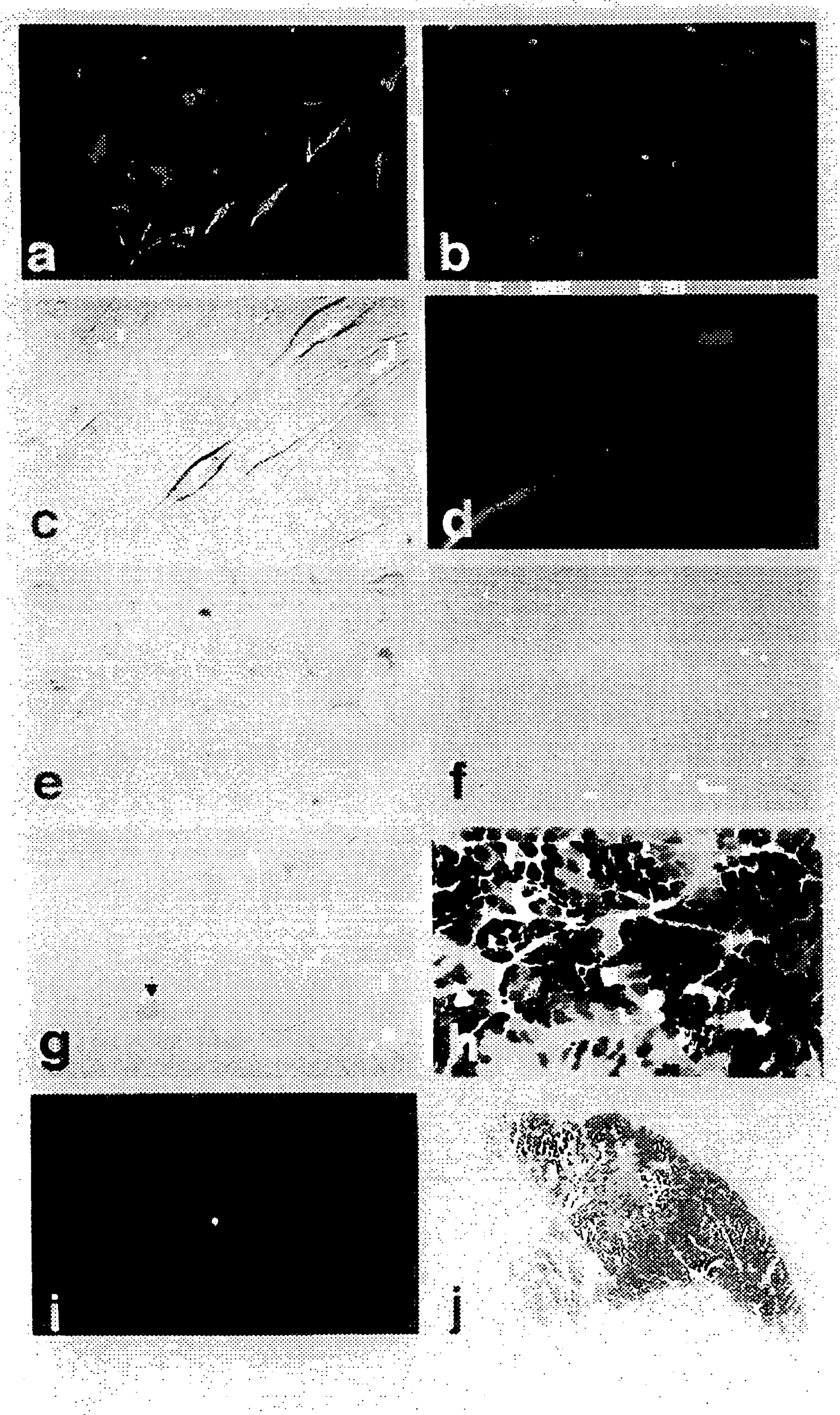


FIG. 8

INTERNATIONAL SEARCH REPORT

Inte onal Application No
/CA 98/01176

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/17 A61K38/18 A61K38/20 A61K35/34 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	QIN L ET AL: "Gene transfer of transforming growth factor-beta 1 prolongs murine cardiac allograft survival by inhibiting cell-mediated immunity." HUMAN GENE THERAPY, (1996 OCT 20) 7 (16) 1981-8. JOURNAL CODE: A12. ISSN: 1043-0342., XP002104189 United States cited in the application see page 1982, column 1, paragraph 1 see page 1986, column 2, paragraph 1 see page 1982, column 1, paragraph 4 ---	1-4,59
X	WO 97 42324 A (SCHERING CORP) 13 November 1997 see page 2, line 3 - line 6; claim 1 see page 2, line 22 - line 30 --- -/--	1,2,4

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 May 1999	10/06/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Charles, D

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Form PCT/ISA/210 (second sheet) (July 1992)

Inte onal Application No
/CA 98/01176

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 28541 A (UNIV LAVAL ;TREMBLAY JACQUES P (CA)) 19 September 1996 cited in the application see page 6, line 13 - line 21 see page 8, line 10 - line 17	1,2, 13-15
X	IKUO KINOSHITA ET AL: "PRETREATMENT OF MYOBLAST CULTURES WITH BASIC FIBROBLAST GROWTH FACTOR INCREASES THE EFFICACY OF THEIR TRANSPLANTATION IN MDX MICE" MUSCLE & NERVE, vol. 18, no. 8, 1 August 1995, pages 834-841, XP000572291 see page 835, column 1, paragraph 2 see page 836, column 2, paragraph 2 see page 839, column 2, paragraph 2	1,2, 13-15
X	WO 93 17698 A (SCHERING CORP) 16 September 1993	1,2,4
Y	see page 1, line 8 - line 11; claims 10,11,13; example 5	5,6
Y	WO 97 36602 A (UNIV LAVAL ;TREMBLAY JACQUES P (CA); ROY RAYNALD (CA); VILQUIN JEA) 9 October 1997 cited in the application see page 10, line 5 - page 11, line 5; claims 1-3	5,6
X	WO 97 05896 A (SCHERING CORP) 20 February 1997 see the whole document	1,56,58
X,P	WO 98 22130 A (SCHEPENS EYE RES INST ;DANA M REZA (US)) 28 May 1998 see page 4, line 7 - page 5, line 3; claims 13,17,18	1,2,4
X,P	MERLY F ET AL: "Anti- inflammatory effect of transforming growth factor-betal in myoblast transplantation." TRANSPLANTATION, (1998 MAR 27) 65 (6) 793-9. JOURNAL CODE: WEJ. ISSN: 0041-1337., XP002104190 United States cited in the application see the whole document	1-4

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Inte onal Application No
/CA 98/01176

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	POWELL W C ET AL: "EXPRESSION OF THE METALLOPROTEINASE MATRILYSIN IN DU-145 CELLS INCREASES THEIR INVASIVE POTENTIAL IN SEVERE COMBINED IMMUNODEFICIENT MICE" CANCER RESEARCH, vol. 53, no. 2, 15 January 1993, pages 417-422, XP002054097 cited in the application see the whole document -----	7-9

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INTERNATIONAL SEARCH REPORT

...ation on patent family members

Int... onal Application No
.../CA 98/01176

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		EP 0896542 A	17-02-1999
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		EP 0845994 A	10-06-1998
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